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EXPERIMENTAL CELL RESEARCH

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THE INFLUENCE OF IONIZING RADIATION ON MITOTIC ACTIVITY IN THE RAT'S CORNEAL EPITHELIUM[1]

J. S. FRIEDENWALD and SYLVIA SIGELMAN

Wilmer Ophthalmological Institute, Johns Hopkins University and Hospital, Baltimore, Md.

Received October 31, 1951

Delay of mitosis following exposure to ionizing radiation has been reported in every type of tissue studied. In view of the universal incidence of this phenomenon and in view of the enormous volume of reports on quantitative studies of radiation effects, it is surprising that the mitotic delay has been subjected to extensive quantitative analysis on only a very few tissues. No attempt will be made here to summarize the literature on this subject which has been reviewed in numerous books, monographs and reviews in recent years. The most detailed quantitative studies are those by Henshaw on the *Arbacia* egg (4, 6, 7), by Carlson and his coworkers on the grasshopper neuroblast (2) and by Strangeways on the chick fibroblast in tissue culture (9, 10).

It is generally agreed that the most significant aspect of ionizing radiation on mitosis is a delay of the entrance of the cell into mitosis, a prolongation of interphase. The tissues listed above that have been extensively studied are all characterized by rapid cell division, that is, by relatively short interphase periods. It seemed possible that a tissue that was steadily growing but nevertheless possessed, normally, a very long interphase, might be particularly sensitive to this type of radiation injury and might, therefore, reveal some new aspects of the radiation effect and possibly, also, of the nature of the process during interphase by which the cell prepares itself for the next mitosis. The corneal epithelium of the rat seemed particularly suitable for study from this point of view. Previous investigations in this laboratory have shown that this tissue has a remarkably steady rate of mitosis (1). The actual histologically visible process of division takes approximately 70 minutes but only about one cell in 200 of the proliferating basal layer of epithelium is normally found to be in mitosis. Consequently the average duration of interphase is of the order of 230 hours.

A preliminary analysis of the duration of mitotic inhibition in various tissues exposed to the same dose of radiation indicates that the effect is in fact correlated with the duration of normal interphase (Table I).

[1] This work was supported in part by a Grant-in-Aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

TABLE I

Author	Tissue	Normal Interphase Minutes	50 per cent Recovery Following 1 000 r Minutes
Henshaw	*Arbacia* first cleavage	?	40
Carlson	Grasshopper neuroblast	30	1 200
Present Study	Rat corneal epithelium	14 000	4 500

The method of mitotic assay used in the present experiments is that previously reported from this laboratory (1). Unless otherwise stated the numbers specified as "per cent mitoses" represent the number of mitoses found in a standard area of the irradiated cornea divided by the number of mitoses found in an equal area of the unirradiated cornea of the same animal. The area in which mitoses were counted was in all cases a narrow meridional strip across the cornea which yields on the average, in normal control animals, approximately 110 mitoses. The meridional strip was chosen for assay since there is a slight variation in the number of mitoses per square millimeter in the periphery of the cornea as compared with the central zone (10–50 per cent excess in the periphery). The area counted represents approximately 1/50 of the total area of corneal surface. The standard deviation in counts between the two eyes of the same animal was found to be ± 13 per cent (1). Of this approximately ± 9 per cent is to be attributed to the size of the samples counted. The biologic variation plus counting errors, therefore, also contribute an uncertainty of approximately ± 9 per cent to the result. The standard deviation can be diminished either by counting mitoses in larger areas per cornea or by averaging the results on a larger number of animals. We have used 4 or 16 animals per experiment in most of the experiments reported below and have found the results reproducible with the standard deviation of approximately ± 15 per cent in the former case, approximately ± 10 per cent in the latter. Such figures include not only the sampling error in the assay but also the experimental error in x-ray dosage and the biologic variation in response of different animals to a given dosage. When a series of experiments is compared in which the time between exposure and removal of the eyes for mitotic assay is varied, smooth curves can be plotted which fit the data within the limits expected from this statistical analysis.

I. *Preliminary Experiments*

The initial experiments to be reported were performed by the late Dr. Wilhelm Buschke who was then a member of our staff, in collaboration with Dr. Gustav Bucky of New York. The purpose of this preliminary study was to discover whether there was any difference in the mitotic effect of low voltage as compared with high voltage x-rays. Under ether anesthesia one eye of each rat was exposed to the radiation, the other eye serving as control. The dosage was varied from 100 to 8000 roentgens. For each dosage level a time series was run so that the shape of the recovery curve could be observed and the time for 50 per cent recovery estimated. Two

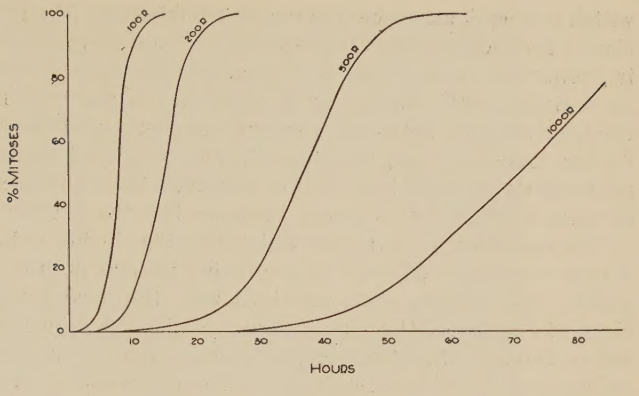

Fig. 1. Recovery from mitosis inhibition following different doses of x-radiation.

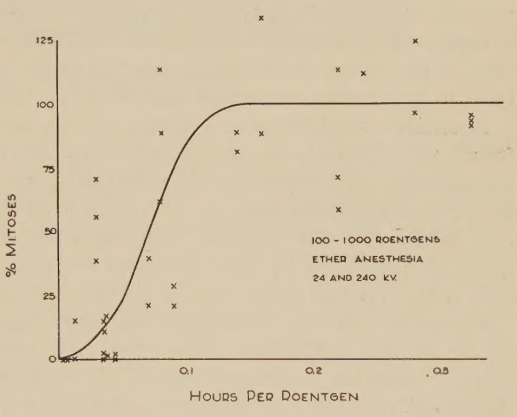

Fig. 2. Recovery from mitosis inhibition following different doses of x-radiation. Number mitoses counted in the exposed eye as compared with the number counted in the unexposed eye is plotted as ordinate. The abscissa is the time following exposure in hours devided by the dose in roentgens. Each point is the average per cent mitoses in two animals with the same exposure and the same time from exposure to removal of the eyes.

animals were used for each observation and the results for each pair averaged. One set of experiments was performed with a 25 kilovolt source and one with a 240 kilovolt source. Calibration of the radiation output was established by Dr. Rosenthal and Dr. Bucky and the exposures were performed in Dr. Bucky's laboratory. The animal's eyes were enucleated at appropriate times, fixed in formalin alcohol and sent to our laboratory for mitosis counting. A total of 80 rats were used in this experiment and five different dosage levels explored (100, 200, 500, 1000, 8000 r).

Since only two animals were used for each observation in this preliminary experiment the sampling error was considerable. Nevertheless the data sufficed to reveal significant trends in the results. No difference was found between the effects of hard and soft x-rays when the same dose in roentgens was given with either of the two sources. With 8000 roentgens severe corneal damage resulted with both radiation sources. With doses up to 1000 roentgens no tissue damage was noted. The following analysis refers only to the results obtained with 1000 roentgens or less.

When for any given dose the course of the inhibition and recovery is studied with respect to time it is found that mitoses disappear completely from the exposed cornea in a short interval after exposure and that recovery follows an S shaped curve

which is steeper and occurs sooner the smaller the dose (Fig. 1). More detailed examination of the recovery curves showed that the time to 50 per cent recovery varied directly in proportion to the dose. We have, therefore, plotted all the data of this experiment on a single chart where the ordinate is the per cent mitoses found, the abscissa is the time in hours between exposure and removal of the eyes for mitotic assay divided by the dose in roentgens (Fig. 2). The S shaped curve drawn in the figure is the probability integral best fitting the data. Though the scatter is considerable no systematic difference was found between the effect of hard and of soft x-rays.

The statistical analysis on which this conclusion is based is as follows. If the hard x-rays were more efficient in producing mitosis inhibition than soft x-rays, then the points representing data obtained with the hard x-rays should tend to fall to the right of and below the S shaped curve while those for the soft x-rays should tend to fall to the left of and above the S shaped curve. We have, therefore, computed the deviations of the two sets of data from the curve. The mean deviation in per cent mitosis for the soft x-rays is $+5$ per cent and its standard error ± 5 per cent. The corresponding figures for the hard x-rays are -4 per cent and ± 6 per cent. In neither case is the mean deviation greater than its standard error. Consequently in neither case is the mean deviation significantly different from zero.

A similar analysis was applied to the data for each dosage level. Again it was found that there is no significant trend for points representing one dosage level to distinguish themselves in distribution from points representing a different dosage level. The statistical analysis of the mean deviation for each dosage level again turned out to be not significantly different from zero. It is concluded that there is no significant difference in the effect per roentgen produced by the hard and soft x-rays and that over the dosage range studied the effect is linearly proportional to the dose.

If we accept the probability integral curve as adequately representing recovery of the mitotic inhibition (except for experimental scattering of the results), then we can say that the recovery time is $0.07 \pm .023$ hours per roentgen. Here the standard deviation of $\pm .023$ is related to the slope of the probability curve and represents the standard deviation in recovery time among the population of corneal cells that were exposed to the radiation. We shall see later, with data based on experiments with larger numbers of animals, that the probability integral does, in fact, fit the experimental data reasonably well.

II. *Dosimetry*

Following these preliminary experiments more extensive studies were undertaken with low and medium voltage x-ray machines. For the low voltage we used a water cooled grenz ray tube with a tungsten target and a beryllium window.[1] The output from this tube was initially calibrated by Dr. Braestrup with a thin walled nylon ionization chamber. It was used at 14 KV and 8 KV input. For most of the experiments the animal was so placed that the eye to be irradiated was protruded with the lids held back and exposed at a distance of 26 cm from the beryllium window (30 cm. from the target). The optical axis of the eye was directed as nearly as possible

[1] Obtained from The International Medical Research Co., Flushing, N.Y.

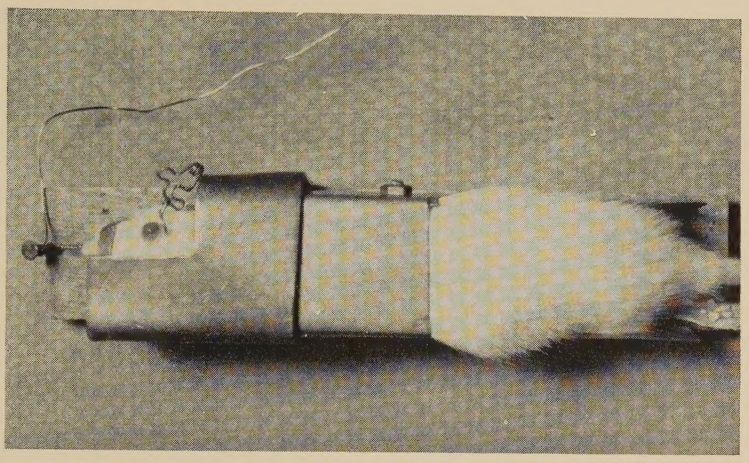

Fig. 3. Holder for rat during exposure.

toward the target (Fig. 3). The unexposed eye was shielded by the animal's head and showed no effects attributable to the radiation even at maximal dosage.

After this tube had been in operation for two years we recalibrated its output with a nylon ionization chamber.[1] The results when corrected for wall absorption by the Bureau of Standards Tables agreed closely with those of the previous calibration. These calibration data yield information as to the intensity of radiation reaching the corneal surface. In view of the theoretical difficulties in calibrating low voltage x-rays an uncertainty of $\pm$ 15 per cent needs to be kept in mind when effects of soft and hard x-rays are compared in roentgen units. Since the nuclei of the mitosing basal layer lie approximately 40 microns beneath the surface, a correction factor for tissue absorption was required. From the calibration data at different distances from the source, after allowance for inverse square of distance, the absorption of the air gap can be computed. With 14 KV input the air absorption between 15 and 20 cms from the window was found to be 7 per cent and that between 20 and 25 cms from the window 7 per cent. Assuming the density of tissue to be 1000 times that of air the 40 microns of shielding tissue at 25 cms from the window would absorb 5.6 per cent of the incident radiation.

Since the rat's cornea covers approximately 115° of the surface of a sphere and the incident ray is approximately parallel to the optical axis of the eye, the rays pass somewhat obliquely through the shielding layer in reaching those basal cells that are near the corneal margin. It was calculated that the shielding effect at the periphery is 2.5 times as great as at the vertex and averaged along a corneal meridian was almost twice that at the vertex or 11.5 per cent of the incident radiation. If the eye is not directed with its optical axis pointing exactly toward the source this extra absorption due to oblique incidence increases somewhat. We estimate that, on the average the error in setting of the eye in respect to the plane of the

[1] This chamber was graciously loaned to us for this purpose by Dr. U. Fano of the Bureau of Standards.

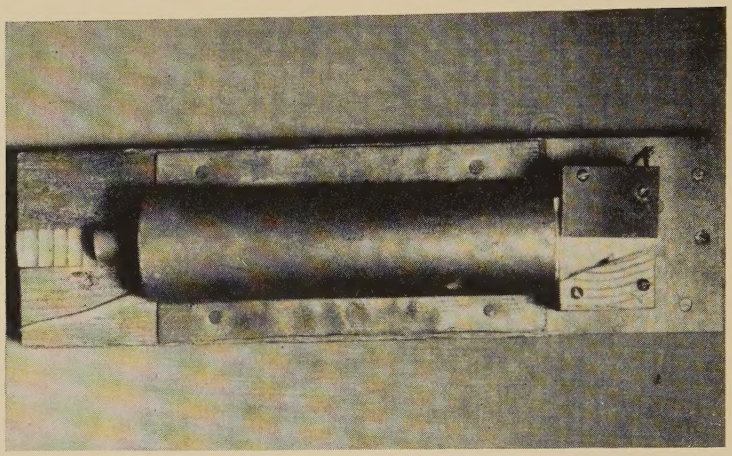

Fig. 4. Holder for unanesthetized rat during exposure.

meridional strip in which mitoses are counted amounts to 5° and that the average correction factor for tissue absorption under the specified conditions amounts to 14 per cent. For 8 KV input and 15 cms air gap a different correction factor needed to be computed. Using the same argument as that just outlined the average absorption of the overlying tissues for these conditions was estimated to be 30 per cent. In the data to be reported below all these corrections have been included.

For medium voltage we have used an air cooled fluoroscopic tube run at 85 KV, 5 milliampere input with 3 mm. aluminum shielding. The calculated absorption of overlying tissue is so slight in this case that experiments can be performed with the eyes closed without requiring an appreciable correction in dosage. For experiments without general anesthesia the rat holder shown in Fig. 4 was used. Most animals were found to keep quiet without struggling for at least 5 minutes when introduced into this holder. In experiments with this source the unexposed eye was shielded by 3 mm. lead. The output of this instrument was measured at the distance used for exposure with a bakelite ionization chamber[1] placed inside the rat holder in the position usually occupied by the rat's head. In spite of occasional incontrollable line voltage variations the output of this source measured in roentgens varied by less than ±5 per cent in 30 half minute intervals tested.

III. *Effect of Dosage, Dosage Rate and Input Voltage*

Experiments were performed under ether anesthesia with the eye protruded. Four or eight animals were used in each experiment. Exposures were made at 8, 14, 85 KV input, and at dosages ranging from 65 to 4000 roentgens. The dosage rate was varied from 6.5 to 430 roentgens per minute.

[1] Two calibrations with two different Victoreens showed that the output was 31 to 33 roentgens per minute at the site where the animals eye was placed during exposure.

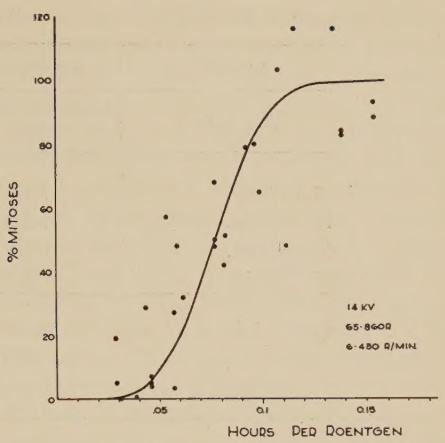

Fig. 5. Recovery from mitosis inhibition following different doses of x-radiation with 14 KV source, plotted as in Fig. 2. Each point is the average of an experiment on four animals. Ether anesthesia, eyes protruded during exposure.

Data obtained with the 14 KV source are shown in Fig. 5. Each point represents the average of measurements on four animals. Per cent mitoses in the treated versus the control eye is plotted versus the time between exposure and enucleation divided by the dose in roentgens. The results confirm the findings in the preliminary experiments that recovery time is proportional to dose. In the experiments charted the dosage ranged from 65 to 860 roentgens, the dosage rate from 6.5 r per minute to 430 r per minute. The curve drawn is the probability integral best fitting the data. The 50 per cent recovery time is approximately 0.08 ± 0.022 hours per roentgen.

The mean deviation of the data from the curve as drawn is -1.5 per cent, the standard deviation of the data ± 16 per cent. In order to see whether the rate at which the dose is given affects the result, we have calculated the mean deviation of the data from the curve for each dosage rate separately. The results are tabulated in Table II. There appears to be a steady progression from positive to negative mean deviations as the dosage rate is increased, suggesting a slight increase in efficiency of the radiation as the dosage rate is increased, but in no case is the mean deviation as great as twice its own standard deviation. It is concluded that the dosage rate within the range tested does not significantly influence the effect.

Similar, though less extensive experiments were performed with 8 KV and 85 KV sources. In Table III we have listed the 50 per cent recovery time found with all the x-ray sources used, including those of the preliminary experiments described above. Considering the wide range covered the results are remarkably uniform. It is concluded that over the range of voltage

TABLE II

14 KV	65–860 Roentgens	6–430 Roentgens per minute	
All data	Mean Deviation −1.5 per cent	Standard Deviation of the Mean	Number of Animals 124
6.5 r/min.	+5.0	± 6.5	12
17 r/min.	+2.5	± 1.4	24
65 r/min.	−3.0	± 2.7	56
430 r/min.	−8.5	±11.5	32

TABLE III

Exposure Under Ether Anesthesia With the Eye Proptosed

Voltage of Source	Number of Animals	50 per cent Recovery time, Hours per Roentgen
8 KV	16	0.11
14 KV	160	0.08
25 KV	35	0.07[1]
85 KV	40	0.08
240 KV	35	0.07[2]

from 8 to 240 KV there is no significant variation in the mitotic effect per roentgen in this tissue.

The strict proportionality of effect to dose extends over the range of 50 to 2000 roentgens. Investigation of the relation of dose to effect at still higher dosage levels is complicated by the inflammatory reaction to radiation damage to the corneal tissues. As a matter of fact moderately severe inflammation of the cornea occurs even with 2000 roentgens exposure, but the inflammatory reaction does not begin until after the recovery from the mitotic inhibition is substantially complete. With 4000 roentgens the inflammatory reaction and the recovery from mitotic inhibition overlap. With this dosage, however, the first half of the recovery cycle can still be followed in relatively uncomplicated fashion and for this portion the recovery curve still conforms to the linear relation characteristic of lesser dosage. With severe inflammatory reaction the corneal epithelium desquamates and may even slough

[1] From preliminary experiments. No correction was made for the absorption of the overlying tissue in estimating the dosage. Such a correction would raise this figure by 10 to 15 per cent.

[2] From preliminary experiments. No correction was made for the deficit in secondary radiation that is to be expected with this high energy source for tissue components close to the surface. The effective dose in these experiments is, therefore, somewhat overestimated and the 50 per cent recovery time per roentgen consequently underestimated.

off from large areas and mitotic counts become erratic. With 8000 roentgens the inflammatory reaction is well advanced before significant mitotic recovery is to be seen.

The strict proportionality between the duration of mitotic inhibition and dose, over this wide range of dosage is in apparent contradiction with the findings reported on many other tissues. In most tissues that have been investigated by others the relationship has been studied by plotting the time for 50 per cent recovery of mitotic activity versus the dose. It was found by these investigators that the duration of the inhibition increases with the dose but that at large dosage levels the increase in the duration of the inhibition is less than proportional to the increase in dosage.

The disagreement is, however, more apparent than real. The declining ratio of 50 per cent recovery time to dose, as the dose increases, has been found on tissues with short mitotic interphases and with dosages so large that the duration of the recovery cycle covers many normal mitotic intervals. Thus those cells which have recovered first are represented by daughter and granddaughter cells before the recovery cycle is complete. The superposition of the mitoses in successive generations upon the recovery curve, necessarily shortens the estimated time at which 50 per cent recovery has occurred. The published data are not sufficient to enable one to calculate a correction factor by which to convert the observed 50 per cent recovery time to an estimate of the time at which 50 per cent of the cells originally exposed would have recovered. However, if one examines the previously published data, one finds that when the observed 50 per cent recovery time is not greater than the time interval of one complete normal mitotic cycle, strict proportionality between dosage and mitotic delay is present. In rapidly mitosing tissues this linear portion is crowded into the first short segment of the curve. The long interphase of the rat's corneal epithelium makes possible the exploration of this linear portion of the curve over a wide range of dosage. The 50 per cent recovery time following exposure to 2000 r is approximately 160 hours, roughly 2/3 of one normal interphase in this tissue.

IV. *Effect of Anesthesia*

The experiments so far reported were performed with the animals under ether anesthesia and the radiated eye protruded with the eyelids held behind the equator of the eye by a small metal clip during the period of exposure. Similar experiments were performed under urethane anesthesia. It was again found that the duration of the mitotic inhibition was proportional to the dosage in roentgens but the shape of the recovery curve was markedly altered. In Fig. 6 are plotted the results of experiments in which 1 gm of urethane was injected intramuscularly per kilo body weight of the rat 3 to 5 hours before exposure to x-radiation. Each point charted respresents the average of results obtained in 4 animals. The ordinate is the per cent mitoses in the exposed as compared with the unexposed eye. The abscissa is the time in hours between exposure and enucleation divided by the dose in roentgens. Exposures were with the 14 KV source and dosage varied from 65 to 2000 roentgens. No significant difference was found between large and

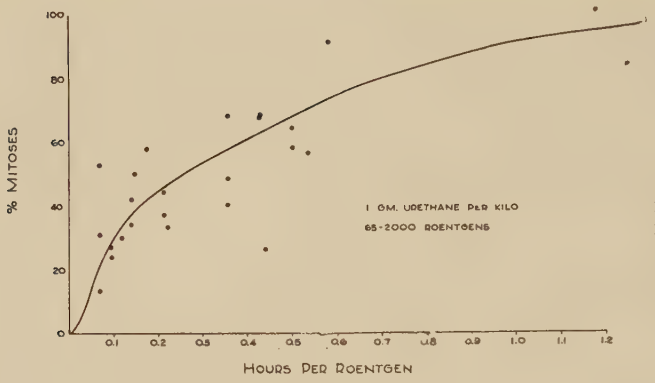

Fig. 6. Recovery from mitosis inhibition following exposure to x-radiation. Urethane anesthesia, eyes protruded during exposure. Each point is the average of an experiment on four animals.

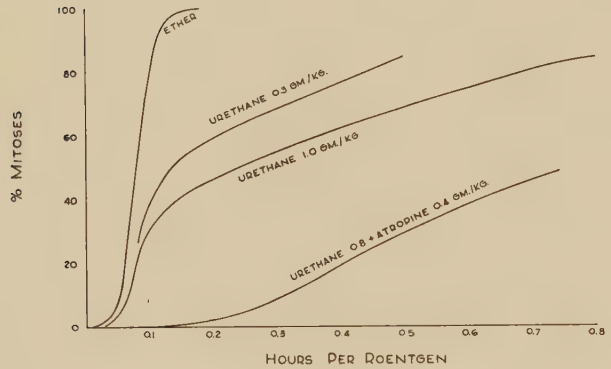

Fig. 7. Recovery from mitosis inhibition for animals under different anesthetics at the time of exposure.

small doses when plotted in this way, i.e. the duration of the effect was proportional to the dose in roentgens.

It is of interest that the mitotic recovery appeared to be independent also of whether or not the animals were under the influence of the anesthetic for a long or short portion of the mitotic recovery. With the dose of urethane given the animals began to stir about approximately 12 hours after the injection, and 24 hours after the injection they appeared normally alert. Mitosis counts in the unirradiated corneas were normal throughout (minor exceptions noted below).[1] For 1000 roentgens the abscissal value of 0.1

[1] In some animals the anesthesia was not complete and these struggled slightly during the period that the eye was held proptosed. Animals showing such incomplete anesthesia if sacrificed an hour after exposure showed at that time some decrease in mitotic counts in the unirradiated eye. Recovery from this occurred within 2 hours after the irradiation. We attributed this transitory inhibition of mitosis in the unirradiated eye to the adrenergic effect of painful stimuli (3). Control experiments showed that this transitory effect could be elicited in one eye by enucleating the other eye under urethane anesthesia without exposing either eye to x-radiation.

represents enucleation 4 days after exposure to radiation. For 65 roentgens the same abscissal value represents enucleation 6.5 hours after exposure.

The shape of the recovery curve following x-ray exposure under urethane is very different from that following exposure under ether. The recovery curve at first parallels that following exposure under ether, but after 40–50 per cent of the initial mitosis rate has been reached further recovery is extremely slow. Recovery curves of similar shape have been reported by Smelser for rat corneal epithelium following exposure under barbiturate anesthesia (8). If lower doses of urethane are used the break in the curve comes at a higher per cent of recovery (Fig. 7). If atropine is injected in addition to urethane the break disappears and the whole recovery cycle is enormously prolonged. Under these conditions mitotic activity in the unirradiated eye is reduced to approximately 50 per cent of the normal during the first day after the injection and declines to still lower levels on the second and third day. Most of the animals died on the fourth day after the injection. With urethane plus atropine the animals did not struggle during the period of proptosis. Control experiments without x-ray exposure in which one eye was enucleated under urethane plus atropine anesthesia showed no transitory decline in mitosis numbers in the other eye during the next hour.

The break in the recovery suggests that urethane makes some cells in the corneal epithelium enormously more sensitive to the effect of radiation. In order to see whether the cells made more sensitive were located in some particular zone of the cornea, mitosis counts were made in equal areas close to the corneal margin, in mid-zone half way between the margin and the center, and in the central corneal area. Twenty animals were chosen for these counts showing approximately 50 per cent recovery of mitosis in the urethane experiments and 20 animals showing approximately 50 per cent recovery following exposure under ether. The results are shown in Fig. 8. The control eyes of the animals that had been anesthetized with urethane failed to show the normal deficit of mitoses in the center as compared with the periphery of the cornea. Relative to the abnormally high counts in the central portions of the control eyes the recovery of mitotic activity was less complete in the central than in the peripheral portions of the irradiated eyes of these animals. The reverse is true for animals that had been anesthetized with ether.

The very marked enhancement of sensitivity to radiation found in the experiments in which the animals received injections of urethane plus atropine deserves some comment. The dose of atropine required to elicit this effect is enormous, far outside the range ordinarily used for anticholinergic

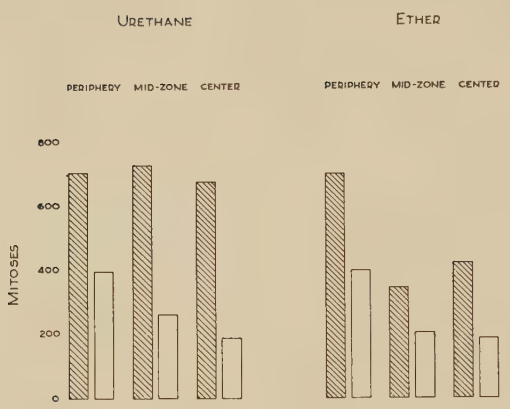

Fig. 8. Mitosis counts in different corneal zones at approximately 50 per cent recovery. Hatched area represents mitoses counted in control unradiated eyes, open areas represent counts in irradiated eyes of the same animals.

TABLE IV

	50 per cent Recovery Time, Hours per Roentgens	
	Eyes Protruded	Eyes Closed
Ether	.08	.053
Urethane plus Atrophine	.56	.37
No anesthesia		.03

effect. It would appear that atropine makes universal the enhanced sensitivity found in a fraction of the cells under urethane. Under urethane plus atropine the recovery curve has an S shaped form. For a given dose of radiation the 50 per cent recovery time under urethane plus atropine is approximately seven times that under ether. Since the mitotic activity in the unirradiated eye is reduced by 50 per cent or more, the enhanced sensitivity found may be attributable in part to a slowing down of mitotic activity during this long lasting pharmacologic effect. Even with due allowance for this possible complication, however, there is a genuine increase in sensitivity under urethane plus atropine as compared with ether.

Effect of Protruding the Eye. The experiments just reported were done with the 14 KV source and the animals eyes opened and protruded during the exposure. Similar experiments with the 85 KV source yielded similar results. When exposures were made with the 85 KV source with the animal's eyes closed the effect per roentgen was reduced. Table IV summarizes the

TABLE V

Exposure to X-rays Under Various Oxygen-Nitrogen Mixtures, Four Animals Used for Each Determination

Per cent 0_2	Per cent Mitoses	
	Ether Anesthesia[1] 48 Roentgens	Urethane + Atropine[2] 5 Roentgens
100	38	49
20	25	65
10	52	—
5	41	53

results under varying conditions. It will be noted that the relative increase in sensitivity under urethane plus atropine is the same with the eyes closed as open. The sensitivity with no anesthesia is less than under ether.

We can offer no explanation for the difference in sensitivity between eyes protruded and eyes closed. Control experiments showed that protruding one eye for 10 minutes under ether anesthesia without exposure to x-rays had no measurable effect on the mitotic activity of the corneal epithelium. The effect cannot be attributed to exposure of the cornea to ether vapor because it occurs also under urethane plus atropine. The screening effect of the closed lids was estimated as negligible for the 85 KV source, and experiments with the eye protruded but screened by 3 mm. lucite showed that the effective dose was reduced by less than 10 per cent by this screening.

The attempt was made to see whether the difference between eyes closed and eyes protruded could be attributed to differences in the partial pressure of oxygen in the corneal tissues. Experiments were performed under urethane plus atropine anesthesia with the exposed eye protruded, the whole animal being placed in a closed chamber through which various mixtures of oxygen and nitrogen were circulated for one minute before exposure and throughout exposure to radiation. Comparisons were made with pure oxygen, air, 10 per cent and 5 per cent oxygen in the gas chamber. Table V shows the results of these experiments indicating that within the range of oxygen pressure used there was no significant difference in the recovery from mitotic inhibition. The partial pressure of oxygen in the corneal tissues could not be measured in these experiments but we feel that the experimental variation was wider than can reasonably be expected between eyes open and eyes closed in air.

[1] Eyes removed 3 hours after exposure.
[2] Eyes removed 2 hours after exposure.

Table VI

Effect of BAL. Right eyes exposed to 32 roentgens, 85 KV source, 3 mm aluminum filter. No anesthesia. Four rats used for each experiment

Hours after Exposure	Per cent Mitosis in Exposed vs. Control Eye		
	Pretreated with BAL	Controls No Pretreatment	BAL instilled after exposure
2	58	45	48
3	102	75	78

In the experiments with the eyes protruded the possible influence of drying of the cornea cannot be excluded. The relative increase in radiation sensitivity, however, was the same eyes open versus eyes closed in experiments with short exposures (eyes open less than one minute) and in experiments with longer exposures (eyes open 10 minutes). Experiments performed with the surrounding air saturated with water vapor at room temperature did not diminish the sensitivity of the protruded eyes. Further study of this intriguing problem seems desirable.

Protective Effect of BAL. The great variation in the effect of x-radiation on corneal mitosis, depending on the anesthetic used and on the conditions of the experiment, strongly suggest that this radiation effect is not to be attributed to the direct ionization of some particular intracellular component directly required in the mitotic process. It would be very difficult to conceive of a target which swells or shrinks depending on the general anesthetic used for the experiment. On the contrary these results suggest that the radiation damage is produced via some chemical vector formed in the cell by absorption of the radiation, the efficiency of such a chemical vector in producing mitotic inhibition being markedly affected by the state of metabolism of the cell.

This conclusion was strongly supported by the finding that the instillation of BAL (dimercapto propanol) onto the cornea before exposure to radiation diminishes the radiation sensitivity. In a series of rats one drop of 10 per cent BAL in peanut oil was instilled into each eye. Immediately after the instillation the rat was introduced into the holder illustrated in Fig. 4 and his right eye exposed to 32 roentgens. Both eyes were removed 2 or 3 hours later. Control animals received the same dose of radiation but no BAL. The results of one such experiment are shown in Table VI. It is evident that the

mitotic inhibition is reduced by the pretreatment with BAL. Treatment with BAL after exposure had no effect. By varying the radiation dosage in the BAL treated animals and in the controls, it was found that in order to produce the same mitotic effect two to three times as many roentgens are required in the BAL pretreated animals as in the controls.

V. *Threshold Effects*

In the preceding sections it has been shown that the degree of retardation in mitosis was linearly proportional to the dose in roentgens over a wide range of dosage, provided the experimental conditions of exposure were kept constant. It is not to be expected that this linear relation would continue to hold as the dosage level is indefinitely reduced. Suppose, for instance, that the dose were reduced to the point at which there is, on the average, one effective ionization per cell. Under these circumstances some cells in the corneal population will receive no effective ionization, some one, some two effective ionizations etc. It is to be anticipated that at this dosage level the frequency distribution of cells receiving different integral numbers of effective ionizations would follow Poisson distribution. Under these circumstances the shape of the recovery curve cannot be expected to follow the simple S shaped course found at higher dosage levels. On the contrary, as the dose is progressively lowered one would expect to find that an increasing fraction of the cells are unaffected by the radiation, and that the 50 per cent recovery time *of those cells that are affected* should not decrease linearly in proportion to the dose but should tend toward an assymptotic level corresponding to the effect of a single effective ionization per cell.

The argument presented in the preceding paragraph is oversimplified in two respects. Carlson (2), and others, have shown that cells in "very early prophase" or what we prefer to call "premitosis" are more sensitive to mitotic retardation than are cells in interphase. Thus when the parade of cells moving toward mitosis is stopped as the result of exposure to radiation the advance guard of this parade, the cells in premitosis, is stopped for a longer interval. Recovery of the cells exposed in premitosis brings them to mitotic activity simultaneously with the less retarded cells that were in late interphase at the time of exposure. Consequently there is a brief period during recovery when these two groups of cells are simultaneously in mitosis. At low doses of radiation Carlson found that this leads to a brief overshooting of the recovery curve above the normal level of mitosis numbers. This overshooting of mitotic activity which becomes visible following exposure to

low doses will, itself, distort the relation between dosage and 50 per cent recovery time as dosage is reduced.

Carlson found that in the grasshopper neuroblast the duration of this hypersensitive "very early prophase" was approximately 15 minutes. Evidence will be given below indicating that in the rat's corneal epithelium the time normally required for cells to pass through this hypersensitive "premitotic" stage is less than half an hour. If none of the cells that were in premitosis at the time of exposure have entered mitosis by the time that 50 per cent of the first echelon of interphase cells have recovered from inhibition, then the time to 50 per cent recovery of interphase cells would be overestimated, at most, by 30 minutes. In so far as the recovery of the cells that were in premitosis at the time of exposure is superimposed upon the recovery of the first echelon of interphase cells this overestimate of the effect of the radiation on cells in interphase at the time of exposure will be reduced. This error in estimating the interphase effect will have to be kept in mind in the analysis of threshold phenomena.

The second oversimplification in the argument presented above is that this argument, and the application of Poisson theory to the data, assumes that ionizations are distributed at random through the cell. Actually the ionizations are expected to occur along tracks in the case of the harder x-rays or in small dense clusters in the case of the soft x-rays. It is conceivable that, as dosage level is lowered, we may reach the point at which there is, on the average, only a single track or cluster per cell or per nucleus. If this should happen then some cells will receive no effective dose of radiation even though the average number of ionizations per cell might still be largely in excess of the threshold at which there is on the average only one effective ionization per cell. A distinction between these two potential types of threshold is possible through a comparison of the dosage levels for hard and soft x-rays at which the threshold appears.

Poisson theory applies equally to the consideration of random distribution of tracks and of random distribution of ionizations. It will be convenient, therefore, to define the threshold dosage as that dose at which $\frac{1}{e}$ or 37 per cent of the cells suffer no mitotic delay. This argument is, plainly, based on the hope that as lower and lower doses are explored a dosage range will be found at which some of the cells show no mitotic delay, and that within this range the quantitative relation to be expected on Poisson theory will be found to hold. Poisson theory would predict that the negative logarithm of the fraction of cells unaffected should be linearly proportional to the dose.

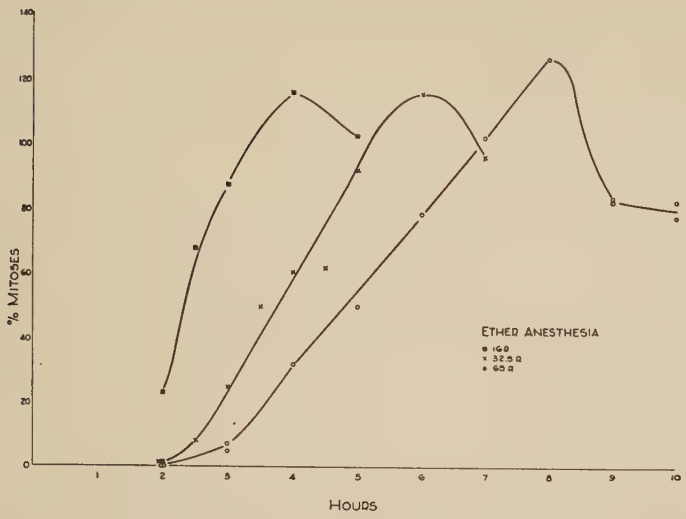

Fig. 9. Overshooting during recovery from mitosis inhibition following exposure to low doses of radiation. Ether anesthesia, eyes protruded during exposure.

Experiments to test this argument were performed under ether anesthesia, under no anesthesia, and under urethane plus atropine.

Exposure under Ether Anesthesia. The use of ether anesthesia is not ideal for exploration of the threshold effect since the anesthesia itself produces a marked but short inhibition of mitosis (1). Recovery from the ether effect is, however, complete by two hours after termination of the anesthesia. By limiting our investigation to periods of two hours or more after exposure to radiation, we were unable to extend our studies to very low doses. The results, however, represent an extension toward the threshold region of the experiments reported in section II above. Exposures were made with the 14 KV source at a dosage rate of 65 r per minute. The eyes were held open and proptosed during exposure.

The results of experiments at three dosage levels are shown in Fig. 9. Each point on the chart represents the average per cent mitosis found in 4 or 8 animals. The results differ in two significant respects from those found with higher doses of radiation. In the first place the overshooting during recovery previously described by Carlson is plainly shown. The overshooting amounts at most to 25 per cent and lasts for less than two hours. It would, therefore, be completely accounted for by the number of cells normally

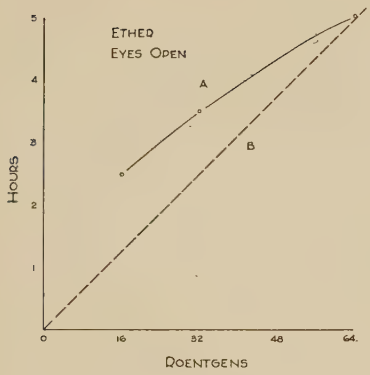

Fig. 10. 50 per cent recovery time following exposure to low doses of radiation. Ether anesthesia, eyes protruded during exposure. Curve A shows the experimental data, the broken line B represents the extrapolation to zero of the linear relation between dosage and 50 per cent recovery time found with higher doses of exposure.

entering mitosis in a one half hour period. This is part of the evidence leading to the conclusion that the duration of the hypersensitive premitotic state is less than half an hour, and hence that the delay in mitosis of cells in interphase at the time of exposure will be overestimated in computations from these curves by less than half an hour.

The second feature to be noted in these low dosage data is that the time to 50 per cent recovery is not proportional to the dose as was found to be the case at higher dosage levels. In Fig. 10 the dotted line represents the extrapolation to zero dosage of the linear proportionality between dosage and 50 per cent recovery time found at higher doses. It will be seen that the 50 per cent recovery time at low dosage is considerably in excess of that predicted by the extrapolation. It would appear that even with the limitations imposed by ether anesthesia the dosage range explored approaches the threshold region.

Exposure Without General Anesthesia. Experiments without general anesthesia were performed with the 85 KV source, with a 3 mm. aluminum filter, at a dosage rate of 32 roentgens per minute. The animals were confined in the holder shown in Fig. 4. The unexposed eye was shielded by 3 mm. lead. The eyes were not proptosed. The results of experiments at several different dosage levels are shown in Fig. 11. Each point charted represents the average result obtained in four animals. It is evident that these experiments extend much farther into the threshold region than those done under ether anesthesia.

In Fig. 12 the times to 50 per cent recovery are charted for the various doses in roentgens. The 50 per cent recovery time is estimated as that time

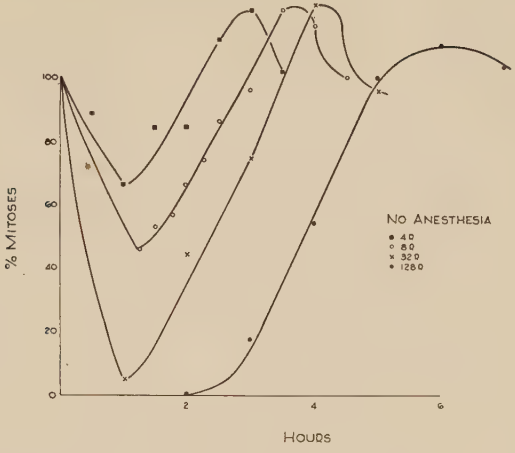

Fig. 11. Recovery overshooting following exposure to low doses of radiation. No anesthesia, eyes closed during exposure, 85 KV source.

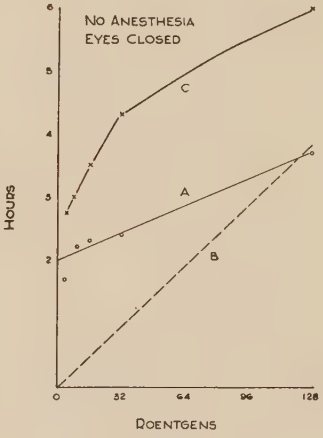

Fig. 12. A: 50 per cent recovery time following exposure to low doses of radiation. No anesthesia, eyes closed during exposure, 85 KV source. B: Extrapolation to zero of the linear relation between dose and 50 per cent recovery time found for higher doses under these conditions of exposure. C: Time to maximum overshooting during recovery.

at which the mitosis rate has recovered half way from its lowest point to normal. The broken line on the chart represents the extrapolation to zero dose of the relation between 50 per cent recovery time and dosage found for higher dosage levels. At 128 roentgens the higher dosage relationship holds, but at the lower doses the 50 per cent recovery time does not tend toward zero but appears to approach a limit of about 2 hours. On this same chart we have also recorded the time between exposure and the peak of overshooting during recovery.

The peaks of the overshooting indicate an excess of approximately 25 per cent over the normal mitotic activity as was also the case in the experi-

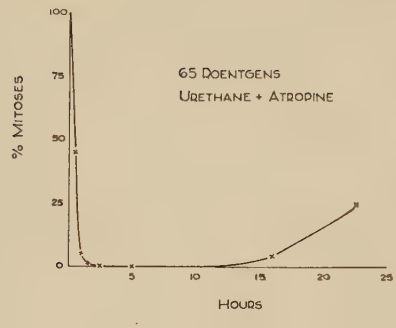

Fig. 13. Per cent mitoses after exposure to 65 roentgens under urethane plus atropine anesthesia. 14 KV source. Eyes protruded during exposure.

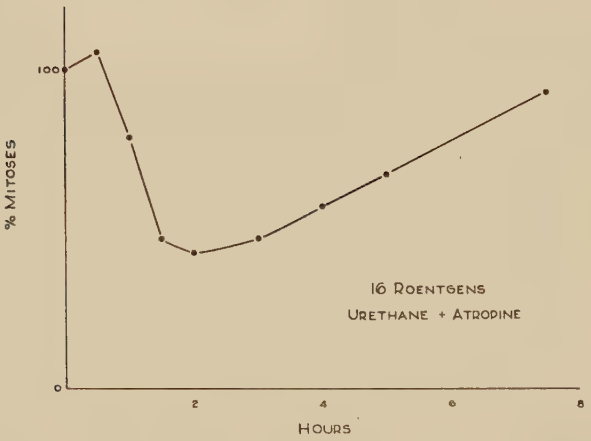

Fig. 14. Per cent mitoses after exposure to 16 roentgens under urethane plus atropine anesthesia. 85 KV source. Eyes closed during exposure.

ments done under ether. The overshooting runs its course within an hour. The total excess of mitosing cells is, therefore, less than the number which normally enters mitosis in the course of one quarter of an hour, and the error in the estimate of the 50 per cent recovery time which this overshooting entails cannot be greater than 1/4 hour.

Exposure Under Urethane and Atropine. The animals used in these experiments received 0.8 gms. of urethane and 0.4 gms. of atropine per kilo by intramuscular injection three hours before exposure to x-radiation. Exposures were made both with the 14 KV source and with the 85 KV source. In some experiments the exposed eyes were held open and proptosed during exposure. A preliminary experiment was performed at 65 r exposure (eyes proptosed) in order to observe in detail the onset of mitotic inhibition under these conditions. It will be seen in Fig. 13 that the number of mitoses

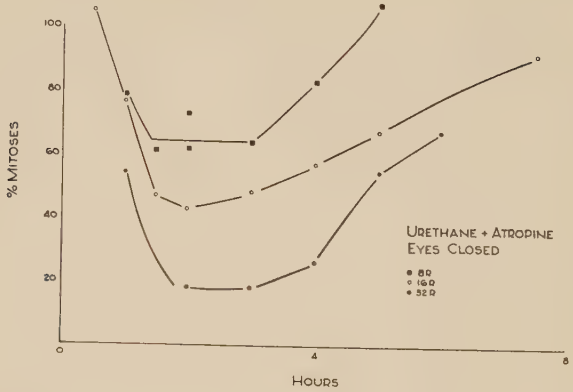

Fig. 15. Per cent mitoses after exposure to 8, 16, 32 roentgens under urethane plus atropine anesthesia. 85 KV source. Eyes closed during exposure.

decreases rapidly following exposure and reaches nearly zero in 70 minutes. Detailed study of these preparations showed that practically all prophases had disappeared 30 minutes after exposure and only a few cells mostly in telophase or reconstruction phase were to be found 60 to 70 minutes after exposure. Since the time required normally for a cell to proceed through the histologically recognizable stages of mitosis in this tissue has previously been found to be 70 minutes, it would appear that urethane and atropine do not interfere with the progress of cells through mitosis and that the onset of inhibition following radiation is immediate.

The most extensive series of experiments was made with the 85 KV source and the eyes closed. In Fig. 14 are charted the results of experiments with 16 roentgens exposure. Each point represents the average found on 16 animals. Several matters of interest are to be noted. At this dose the inhibition following radiation does not make its appearance until half an hour has elapsed after exposure. In this and in other experiments with still smaller doses of radiation no recovery overshooting was noted. It would appear that under urethane plus atropine the relative sensitivity of interphase and premitosis has been reversed and that at 16 roentgens we are substantially below the threshold for cells in premitosis. This unexpected finding considerably simplified the interpretation of the results under these special conditions.

A second point to be noted is the fact that during the interval between 1 1/2 and 3 hours following exposure the per cent mitoses found remains substantially stationary. Under the conditions of these experiments we have a broad zone in which estimates of the per cent of cells unaffected by the radiation can be made. Fig. 15 shows the results of similar experiments at

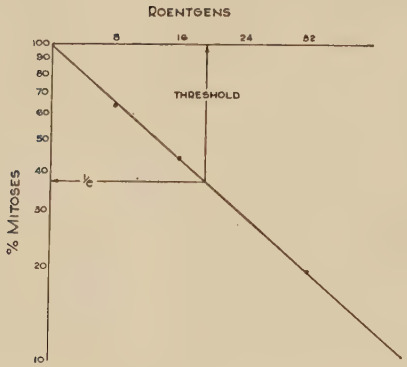

Fig. 16.

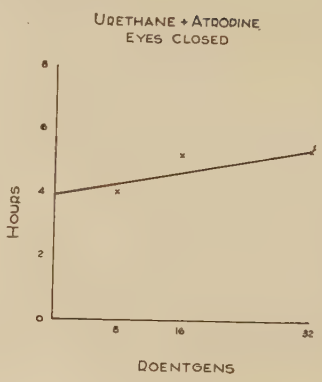

Fig. 17.

Fig. 16. Logarithm of per cent of cells unaffected by the radiation plotted versus dosage.
Fig. 17. 50 per cent recovery time following exposure to low doses of radiation. Urethane plus atropine anesthesia, eyes closed during exposure.

three different dosage levels. Taking the average of the readings between 1 1/2 and 3 hours after exposure for each dosage level, we have charted in Fig. 16 the logarithm of the per cent of unaffected cells versus the dose. The data fall very well on a straight line indicating the Poisson theory is applicable. It is estimated that under the conditions of these experiments the threshold, i.e. the dose at which $\frac{1}{e}$ of the cells are unaffected, is at 19 roentgens.

In Fig. 17 we have plotted the 50 per cent recovery time versus the dose, leading to an estimate of 4 hours as the minimal recovery time under the conditions of this experiment. Since the mitotic rate in the control unirradiated eyes of these animals is very nearly half that in the unanesthetized animal, this estimate of the recovery time for minimum injury is in substantial agreement with the corresponding estimate of 2 hours recovery time from minimal injury in the unanesthetized animal.

Poisson theory enables one to estimate for any given dose the per cent of cells receiving 0, 1, 2, 3, etc. units of injury. Assuming that successive increments of recovering cells occur at 4, 8, 12, etc. hours after exposure one gets a stepwise representation of the recovery process (Fig. 18, line A). Since after large doses of radiation recovery follows an S shaped curve, we have substituted for the stepwise jumps probability integral curves B having the same relationship of spread to 50 per cent recovery as that shown in Fig. 5. Finally we have summed the successive probability curves for each dose arriving at the curve C shown in Fig. 18. In Fig. 19 three curves

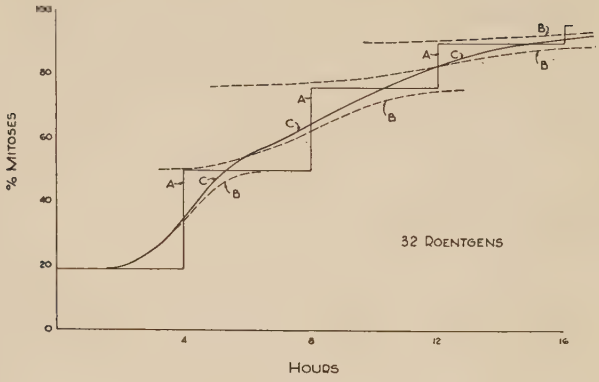

Fig. 18. Construction of theoretical recovery curve for 32 roentgen dose, see text.

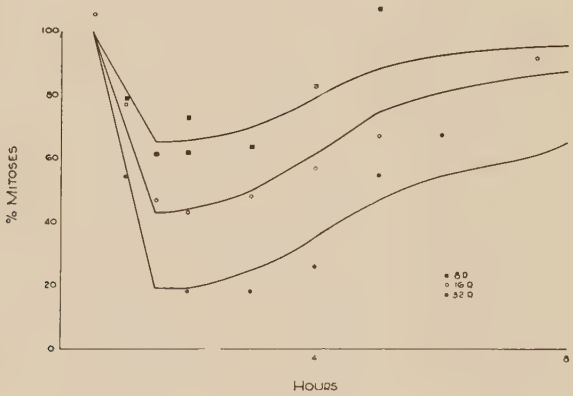

Fig. 19. Data of Fig. 15 plotted in relation to theoretical recovery curves, see text.

of type C corresponding to the three dosage levels used are shown in relation to the experimental data. These curves are derived from experimental estimates of three parameters: the exponential constant of the Poisson equation estimated from Fig. 16; the time of recovery per unit damage estimated from Fig. 17; and the slopes of the probability integral curves estimated from Fig. 5. The standard deviation of the data from the curves is 7.5 per cent. Since each datum is the mean of the results obtained on 16 animals, it is possible to calculate the standard deviation of each of these means. The mean of these standard deviations is found to be 6 per cent. The theoretical curves therefore fit the data within the limits of accuracy of the data.

Experiments were performed under urethane plus atropine with the eye proptosed during exposure. Exposures were made over the range of 4 to 32 r with the 14 KV source and at 16 r with the 85 KV source. The animals were

sacrificed two hours after exposure. Six experiments were performed each involving 16 animals. The resulting data were plotted as in Fig. 16 and led to an estimate of the threshold dose under these conditions as 12 roentgens. Experiments with longer intervals between exposure and removal of the eyes indicate in this case also a 50 per cent recovery time for minimal injury of approximately 4 hours. No significant difference was found between the results with the two sources.

Ionizations versus Tracks. At the beginning of this section it was pointed out that Poisson theory could be expected to hold if dosage levels were reached at which there was either approximately one effective ionization per cell or approximately one ionization track within some postulated sensitive volume. With the eyes proptosed the threshold is found to be 12 roentgens. We estimate the volume of the basal cells in the rat's cornea to be 800 cubic microns and that of their nuclei to be 140 cubic microns. With an exposure of 12 roentgens one would, therefore, estimate an average of 19 000 ionizations per cell and 3 300 per nucleus. If the threshold signifies the presence of one effective ionization the efficiency of the ionization would be $\frac{1}{19\,000}$ per cell or $\frac{1}{3\,300}$ per nucleus. We have no way of deciding from our data whether the ionization must be in the nucleus to be effective. The experiments reported many years ago by Henshaw (5) and also by Zirkle (11) suggest that at threshold doses the ionization must be in the nucleus to be effective.

In order to explore the question as to whether the threshold occurs at a dosage level at which there is a single track per nucleus, some assumptions regarding the geometry of the tracks are required. For the 14 KV source with 26 cms. air gap we have estimated that the average energy of the photoelectrons produced in the tissue is 12 KV and that each track contains approximately 400 ionizations. If these tracks were straight they would be approximately 3.4 microns in length but, owing to the high angle of deflection for collisions at these relatively low energies the path of the photoelectron is far from a straight line. We have assumed, therefore, that the ionization "track" for this source is, on the average, included within a volume of $1 \times 1 \times 2$ microns. On the basis of this assumed geometry one can estimate that, at 12 roentgens dose, there would, on the average, be 8 ionization tracks whose centers lie within the nucleus and an additional 6 partially penetrating tracks whose centers lie outside the nucleus, a total of 14 tracks

penetrating the nucleus. The same argument leads to an estimate of 55 tracks per cell.

For the 85 KV source with 3 mm. of aluminum shielding we have estimated that the average energy of the absorbed radiation is 55 KV, of which approximately 60 per cent will appear as photoelectrons with an average energy of 55 KV and 40 per cent as recoil electrons with an average energy of 11 KV. Thus 40 per cent of the energy can be assumed to be distributed in tracks similar to those obtained with the low voltage source and 60 per cent in tracks that are long compared to the dimensions of the cell with an average density of 34 ionizations per micron. The average length of a photoelectron track passing through a nucleus is estimated as 4 microns and that passing through a cell as 8 microns. Consequently we can estimate that at 12 roentgens with the 85 KV source there will on the average be 15 photoelectron tracks and 6 recoil electron tracks per nucleus making a total of 21 tracks per nucleus. The same argument leads to an estimate of 64 tracks per cell. The threshold for both hard and soft radiation is, therefore, far higher than that at which there is a single track per cell or even per nucleus.

The argument we have pursued leads us back to the first suggestion, namely that the threshold occurs at a dosage level at which there is, on the average, one effective ionization per cell. With the 14 KV source the ionizations occur in densely packed clusters. With the 85 KV source the ionizations are much more widely distributed within the tissue. It follows that, at the same dosage in roentgens there is with the 14 KV source a much larger fraction of cellular volume that is not close to an ionization than is the case with the 85 KV source. Since the effect of the radiation with these two sources is the same per roentgen, we are forced to suggest that the effect can be produced in regions within the cell that do not contain an ionization track.

In the preceding section we have already presented evidence indicating that the effect we are studying is mediated through the production of some reactive chemical agents by the ionizing radiation. The present analysis is in accord with this conclusion and suggests in addition that the radius of effective action of such reactive compounds may be of the order of microns. This is a far larger estimate of effective range than that which has in the past been assumed to hold for the free radicals that are presumed to be the initial chemical products of ionization. The present study provides no basis for the resolution of this paradox. It may be pointed out, however, that the combination of free radicals may yield some relatively stable peroxides capable of diffusing considerable distances within the cell before they are destroyed by reaction with cellular components. It is also possible that the

units of the intracellular material injured by the radiation represent quite large organized aggregates. Under this last assumption a very large effective range need not be postulated for the chemical vector.

VI. *Summary of the Data*

This work has been directed toward a quantitative analysis of the effects of ionizing radiation on mitotic activity in the rat's corneal epithelium. The results reported above may be summarized as follows:

(a) After exposure to x-radiation cells already in mitosis continue through the cycle in an apparently normal fashion, but no new cells enter mitosis until the inhibition begins to pass off.

(b) Plotting mitosis numbers versus time following irradiation shows that recovery follows an S shaped curve. The duration of the inhibition is linearly proportional to the dosage in roentgens over the range 65–4000 roentgens. It is independent of the x-ray voltage over the range 8–240 KV. It is independent of the dosage rate over the range 6.5 to 430 roentgens per minute. All these results can be fitted to a single probability integral curve if the per cent mitoses in the treated as compared with the untreated control eye of the same animal is plotted versus time following exposure devided by the dose in roentgens. This may be summarized in the statement that the inhibition of mitosis lasts 0.08 ± 0.022 hours per roentgen, where the standard deviation refers to the variation in the recovery time among the cells of the corneal population. These results concern experiments in which the animals were anesthetized with ether and had their eyes proptosed during exposure to the radiation.

(c) The duration of the inhibition following exposure is very markedly influenced by the anesthetic used during the irradiation. With no anesthesia it is shortest, with ether anesthesia it is longer, with urethane or barbiturate anesthesia it is considerably longer, with urethane plus atropine it is longest of all. After exposures under urethane the recovery curve is not S shaped but the duration of the inhibition is nevertheless proportional to the dosage over the range 65–2000 roentgens.

(d) The duration of the inhibition following exposure is decreased by pretreatment of the eye with BAL (dimercapto propanol).

(e) The duration of the inhibition following irradiation is greater if the lids are held open and the eye proptosed during irradiation. This difference between open and closed eyes is not attributable to the screening effect of the lids, nor to a local effect of ether vapor, nor to the partial pressure of

oxygen in the tissues during exposure. It is probably not due to drying of the cornea. Further study of this curious phenomenon is indicated.

(f) When animals without anesthesia or under ether anesthesia are exposed to very low doses (30 roentgens or less) the duration of the inhibition does not decline in proportion to the dose but tends toward a minimum value of about 2 hours. At the same time the inhibition becomes less complete, an increasing fraction of the cells being apparently unaffected by the radiation. In addition there appears at this dosage level an overshooting of mitotic activity during recovery, — that is for a brief period toward the end of the recovery cycle the number of mitoses in the exposed eye is greater than that in the unexposed control eye of the same animal. Similar overshooting has previously been described by Carlson following exposure of grasshopper neuroblasts to low doses of radiation. The phenomenon has been attributed to excessive sensitivity of cells in very early prophase (premitosis) to the radiation effect, an interpreta ion which accords well with our data.

When the same experiments are performed under urethane plus atropine anesthesia this phenomenon of recovery overshooting disappears. Under these circumstances with doses of 30 roentgens or less the decline in mitosis number does not begin until 20–30 minutes after exposure. It would appear that under these conditions the cells that are in premitosis at the time of exposure are relatively more resistant to the radiation effect than are those cells that are in interphase at the time of exposure. As the dosage is lowered under these conditions an increasing per cent of the cells show no inhibition of mitosis. The negative logarithm of the per cent of cells unaffected by radiation is, in these experiments, linearly proportional to the dosage, conforming to the expectations on Poisson theory and on the assumption that in this dosage range the average number of effective ionizations per cell is near one. Under these same conditions the 50 per cent recovery time of the fraction of cells affected does not decline toward zero with decreasing dose but tends toward a minimum which we estimate as approximately 0.01 of the duration of interphase in the unirradiated animal.

VII. *Argument*

(a) The sensitivity of the rat's corneal cells to ionizing radiation varies markedly with respect to the general anesthetic administered prior to exposure to radiation. It is difficult to suppose that an intracellular target swells and shrinks depending on the general anesthetic used. Consequently these results appear incompatible with the target theory of radiation effect. They are

compatible with the theory that the biological effect of the radiation is mediated by a chemical vector since the efficiency of an oxidizing agent produced by the ionization would be expected to vary with the metabolism of the cell. This conclusion is strongly supported by the finding that BAL (dimercapto propanol) when administered to the eye before the exposure to radiation has a marked protective effect.

(b) Under similar experimental conditions the effect per roentgen is the same for soft and for hard x-rays. Since, under these conditions, a much larger fraction of the cell volume is devoid of ionizations in the case of soft x-rays than in the case of hard x-rays, it is to be concluded that the chemical vectors which mediate this biological effect are able to diffuse sufficient distances within the cell to compensate for the different degrees of inhomogeneity in the distribution of ionization with soft and hard x-rays.

(c) On comparing urethane plus atropine anesthesia with ether anesthesia or no anesthesia it is found that the former increases the sensitivity of cells in interphase to the radiation effect while it diminishes the sensitivity of cells in premitosis. It would appear from this that the mitotic delay produced by radiation involves different mechanisms in cells which are in premitosis during exposure, from those mechanisms involved in cells which are in interphase during exposure.

(d) Effects are noticeable down to doses as low as 4 roentgens. This corresponds to a concentration of the postulated chemical vectors as low as 10^{-9} M. Since the cell contains many reactive substances in concentrations very much higher than this (for instance SH groups at approximately 10^{-3} M), the chance of damaging an appreciable fraction of molecules of the substance or substances specifically needed for entrance of the cell into mitosis must be exceedingly small. It must be supposed, therefore, that the cell requires a specific amount of this critical material in order to be ready for mitosis and that the damage of even a single unit of this substance causes a delay in mitosis until that unit has been repaired or replaced.

(e) At doses of radiation above the threshold the duration of the inhibition is proportional to the dose, i.e. the *rate* of recovery is independent of the damage. From this it is to be concluded that the critical material is being manufactured at a steady rate independent of the injury, i.e. that recovery occurs by replacement not repair.

At doses of radiation appreciably above the threshold the inhibition is followed by recovery without overshooting. It is to be concluded that the effect of the radiation is to stop the whole parade of cells moving through interphase toward mitosis, and that every cell is equally retarded (except for

the inherent variability of ± 25 per cent in the recovery rate of the cell population). It follows that the critical substance is present throughout interphase and is steadily being manufactured by the cell throughout interphase.

Since during interphase the cell is preparing to become two cells, the amount of the critical material must at least be doubled during this interval, — assuming that this substance is necessary for, and perhaps to some extent used up during mitosis. The duration of the inhibition is, however, linearly proportional to the dose even with doses that produce an inhibition lasting longer than one normal interphase. Consequently, on the basis of the above assumptions, the critical substance must be manufactured at an exponentially steady rate during interphase.

(f) Investigations in the threshold region show that the minimum duration of inhibition in an affected cell is approximately .01 of the duration of interphase, and that as the dose is decreased an increasing proportion of the cells are unaffected. The quantitative relations between per cent of cells unaffected, the 50 per cent recovery time of affected cells, and the dosage, analyzed with the aid of Poisson theory lead to the conclusion that the threshold (i.e. the dosage at which $\frac{1}{e}$ of the cells are unaffected) corresponds to an average injury of one unit of the critical material per cell. It follows that the last unit of this material needed for the cell to be ready to enter mitosis requires .01 of the interphase interval for its manufacture.

(g) On the above assumption it is possible to estimate within limits the number of units of the critical substance present in a cell of this tissue when the cell is ready for mitosis. The calculation is as follows:

1) $$\log Y = \log n + BT$$

where: Y is the number of units present at any moment, n is the number of units present at the beginning of interphase and cannot be less than one, B is a constant which cannot be less than 0.301 (that is not less than the log of 2 since the amount must at least double during interphase), T is the time since the beginning of interphase measured in such units that at the end of interphase $T = 1$, N is the number of units present at the end of interphase.

2) When $T = 1$ $\log Y = \log N = \log n + B$

3) When $T = 0.99$ $\log Y = \log (N - 1) = \log n + 0.99\ B.$

Subtracting (3) from (2) we obtain:

$$4) \qquad \log \frac{N}{N-1} = 0.01\ B.$$

Now, considering the limits between which N must lie, as larger values of N are considered the fraction $\frac{N}{N-1}$ decreases toward unity, and $\log \frac{N}{N-1}$ decreases toward zero. Consequently, as N increases, B must decrease. We have, however, already assumed that Y must at least double during one interphase and therefore that B cannot be less than 0.301. Placing this minimum value of B in equation 4) will, consequently, yield the upper limit for N compatible with our assumptions. Performing this operation gives N_{max} approximately 140. If on the other hand we eliminate B from equations 2) and 4) we obtain:

$$5) \qquad \log N = \log n + 100 \log \frac{N}{N-1}.$$

In this equation N increases with increasing n. We have, however, already assumed that n cannot be less than unity. Inserting $n = 1$ in equation 5) will, consequently, yield the lower limit of N compatible with our assumptions. Performing this operation gives N_{min} approximately 40. The order of magnitude of N determined above is of possible interest since, in the rat there are 48 chromosomes per cell. It seems appropriate to suggest, most tentatively, that a cell may be ready for mitosis when it contains one or two units of what we have called the critical substance per chromosome.

CONCLUSIONS

The study of mitotic inhibition in the rat's corneal epithelium by ionizing radiation indicates:

1) That the inhibition of mitosis by ionization radiation results from the production in the tissue of reactive chemical substances and is markedly influenced by the conditions of the experiment.

2) That mitosis is inhibited through the destruction of some substance of which the cells requires a specific quantity in order to be ready for mitosis, and which the cell manufactures at an exponentially steady rate during interphase.

3) It is estimated that the corneal epithelial cells of the rat require between 40 and 140 units of this critical material in order to be ready for mitosis.

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A METHOD FOR COUNTING MITOCHONDRIA IN TISSUE HOMOGENATES

EMMA SHELTON, W. C. SCHNEIDER, and MARY JO STRIEBICH[1]

National Cancer Institute, Bethesda, Md.[2]

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The finding that relatively homogeneous preparations of mitochondria could be isolated in good yield from homogenates of mammalian tissues in sucrose solutions (6) has resulted in the widespread study of these intracellular organelles and their biochemical properties (16). Such studies would be considerably implemented if an accurate method were available for the enumeration of mitochondria. The method would provide a specific index for determining the recovery of mitochondria in the isolated mitochondrial fraction as well as the degree of contamination of other cell fractions and would provide an estimate of the variation in the number and composition of these bodies under different physiological and pathological conditions.

In a recent publication (1), Harman described a method for counting mitochondria which consisted of spreading a measured drop of a suitably diluted mitochondrial preparation over a measured area of a microscope slide, fixing the smear in osmium tetroxide vapor and staining it with a differential stain. The stained mitochondria were then counted with the aid of a Whipple disc and the number present in the original, undiluted suspension was calculated. The accuracy of the method was not determined.

The present paper describes a method of determined accuracy for counting unfixed, unstained mitochondria in a Petroff-Hausser bacteria counting chamber by means of a phase microscope. Estimates of the number of mitochondria in homogenates of tissue can routinely be obtained within as little as one hour after an animal is killed.

MATERIALS AND METHODS

Mitochondria were counted in dilutions of whole tissue homogenates which were prepared in 0.25 M sucrose from the unperfused livers of C_3H mice, about 3 months

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of age and both sexes, in a modified Potter-Elvehjem homogenizer (plastic pestle, smooth-wall test tube). All procedures were carried out in the cold, except for the actual counting.

Aliquots were removed from the homogenate and diluted 1:150, 1:300, 1:450, or 1:600 with 0.125 M sucrose solution according to the concentration of the homogenate. These dilutions were adjusted so that about 4–8 mitochondria were counted per microscope field because this was the maximum number that could be counted accurately. In the first experiments, the dilutions were made in two steps so that a 1:300 dilution was prepared by mixing 0.2 ml of the homogenate with 9.8 ml of 0.125 M sucrose solution and then adding 1 ml of this mixture to 5 ml of 0.125 M sucrose solution. Later dilutions were prepared in one step by adding 0.2 ml, 0.1 ml, and 0.05 ml of the original 5, 10, and 20 per cent homogenates to 29.8 ml, 29.9 ml, and 29.95 ml of 0.125 M sucrose solution, respectively.

To prepare the Petroff-Hausser bacteria counting chamber[1] for receiving the sample to be counted, the reinforced cover slip was rubbed into close contact with the chamber's supporting ribs and a sample of the diluted homogenate was introduced under the cover slip by means of a pipette which was filled with a rubber bulb. The pipettes were made from pyrex glass tubing, measured 6 cm overall and had a blunt, thick-walled tip with a fine (but not capillary) opening. Since it was far easier to fill the chamber outside of its bakelite holder, the holder was converted from a square to a "U" shape by cutting away the front side and thus the chamber could be slid into the grooves of the holder without disturbing the cover slip. After a count was made, the chamber and cover slip were washed in a mild detergent and thoroughly rinsed and dried. The chamber was cleaned with potassium bichromate-sulfuric acid cleaning solution whenever necessary in order to prevent the formation of fine bubbles in the counting area. When the chamber was properly filled, Brownian movement of the mitochondria was confined to a circumscribed area, but in an improperly filled chamber, the mitochondria streamed across the field and could not be counted. The counting was done with Bausch and Lomb and AO Spencer phase contrast microscopes (10 × ocular and 1.8 mm oil immersion dark contrast objective).

The ruled grating in the bacterial counting chamber is similar to that of a hemocytometer, but in counting the mitochondria, only the center square millimeter was used. This square is divided into 25 groups of 16 small squares and with the magnification used, the microscope field conveniently covered 4 small squares. Twenty small squares (5 groups of 4) were counted in the corners and the center of the square millimeter. This gave a total count of 100 small squares or 25 microscope fields covering a representative area.

Since the depth of the cell is 0.02 mm and each small square is 0.05 mm on a side, the volume over each small square equals 5×10^{-5} cu. mm, or the volume over 20 small squares was 10^{-3} cu. mm. The number of mitochondria in the 5 groups of 20 small squares was recorded and the average number of mitochondria per 10^{-3} cu. mm was determined. Calculation of the number of mitochondria per cc. of whole homogenate was then made using the following formula:

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[1] An improved chamber adapted for counting mitochondria can be obtained from the Arthur H. Thomas Company, West Washington Square, Philadelphia 5, Pennsylvania.

Average number of mitochondria per 20 small squares × dilution used

$\times 10^6$ = Mitochondria/cc. homogenate.

Identification of mitochondria. The recognition of mitochondria in dilutions of whole homogenate or in preparations of isolated mitochondria was of primary importance to the validity of the counting method. In order to become acquainted with mitochondrial morphology, an homogenate was diluted 1:50 with 0.25 M and 0.125 M sucrose solution, and with distilled water. These dilutions were examined with the phase microscope. The mitochondria in 0.25 M and 0.125 M sucrose solution appeared as spheres with a diameter ranging from 0.5 μ to 3.0 μ. Most of the mitochondria, especially the smaller ones, were solid gray-black, but some appeared as opaque, gray spheres with a black cap or crescent as described by Harman (2). When the homogenate was diluted with distilled water, all of the mitochondria swelled and became greatly reduced in contrast. After careful examination under these different conditions, it was possible to distinguish with confidence between mitochondria and cytoplasmic debris in dilutions of the homogenate in 0.125 M sucrose solution. Isolated mitochondrial preparations treated in a similar manner showed essentially the same morphologic behavior.

RESULTS

Evaluation of errors of method: A preliminary theoretical estimate was made of the number of mitochondria that would have to be counted in order to reach a desired level of accuracy. The statistical method for arriving at this number has been reported in detail by Sanford *et al.* (11). It was found that at least 370 mitochondria would have to be counted to obtain an accuracy of 90 per cent, 95 per cent of the time. The technical aspects of counting mitochondria imposed a limit on the number of mitochondria that could be counted, so that in one chamber it was possible to count only 100 small squares at the rate of 1–2 mitochondria per square. Therefore, in order to count 370 mitochondria, more than one chamber had to be filled.

Before a reasonable estimate of the accuracy of the method could be made, it was necessary to check three sources of error, namely; 1) chamber to chamber variation, 2) dilution to dilution variation, and 3) homogenate to homogenate variation. Accordingly, an experiment was designed in which not less than 370 mitochondria were counted in at least two separate dilutions made from each of ten homogenates. A sample count from one homogenate is shown in Table I, and the mitochondria per cc. homogenate calculated from all ten homogenates is found in Table II.

These data showed that the mitochondria in any one chamber closely followed the Poisson distribution, and that in any single dilution the chamber to chamber variation was slightly, but not significantly smaller than theory

TABLE I

Complete mitochondria count on homogenate VI (see Table II) showing the distribution of the mitochondria in the 5 groups of 20 small squares in each chamber (column 3). Four 1:300 dilutions (column 1) were made from this homogenate using a two step dilution.

1	2	3					4	5	6
Dilution Number	Chamber Number	Number mitochondria in 20 small squares					Total Counted	Average per 10^{-3} mm^3 volume	Average per ml. homogenate
		1	2	3	4	5			
1	1	28	26	25	28	27	144	28.8	8.6×10^9
	2	27	34	29	16	25	131	26.2	7.8
	3	32	31	28	35	19	145	29.0	8.7
2	1	15	22	18	24	25	104	20.8	6.4
3	1	39	20	29	28	32	148	29.6	8.8
	2	17	25	17	21	21	101	20.2	6.1
4	1	14	18	23	26	23	104	26.8	6.4

Total = 877 Average = 25.1 Average = 7.5×10^9

indicated. The accuracy of the method was considerably enhanced by the fact that freshly prepared mitochondria do not clump in sucrose solution (6), indeed, the fact that the reproducibility of the counts between chambers was better than expected indicated that there was some additional factor present which tended to keep the mitochondria apart. The greatest variation in counts was found to occur between different homogenates, with smaller variations occurring between different dilutions of the same homogenate. The following formula for the per cent error of an average mitochondria count was derived from the data in Table II:

$$\text{per cent error} = \sqrt{\frac{(100)^2}{N} + \frac{(5.5)^2}{D} + \frac{(19.1)^2}{S}}$$

where N = Number of mitochondria counted, D = Total number of dilutions prepared and S = Number of separate homogenates.

As calculated from the data in this experiment, the factor 5.5 is the coefficient of variation between dilutions and 19.1 is the coefficient of variation between homogenates, assuming that each homogenate is prepared from the liver of a single animal. As more data accumulate, these factors undoubtedly will change slightly.

TABLE II

Summary of mitochondria counts on the 10 per cent C_3H mouse liver homogenates in 0.25 M sucrose, diluted 1:300 with 0.125 M sucrose solution.

Homogenate Number	Number of livers pooled	Number of dilutions counted	Number of chambers counted	Total number mitochondria counted	Average number mitochondria per 10^{-3} mm volume	Average number mitochondria per cc. homogenate
I	1	3	5	544	21.7	6.5×10^9
II	2	2	5	643	25.7	7.7
III	2	3	8	1 081	27.0	8.1
IV	2	2	5	798	31.9	9.6
V	2	2	4	653	32.7	9.8
VI	2	4	7	877	25.1	7.5
VII	2	2	5	730	29.2	8.8
VIII	2	3	6	886	29.5	8.9
IX	2	3	5	476	31.7	9.5
X	2	2	4	683	34.2	10.3

Average = 8.7×10^9
10 mice

The value of the formula lies in giving an estimate of how an experiment should be set up to give the desired level of accuracy. It can be seen immediately that the size of the per cent error in any given experiment is largely controlled by the factor $\frac{(19.1)^2}{S}$. The magnitude of this factor can be decreased either by increasing the number of animals whose tissues are pooled in one homogenate (in which case, 19.1 is divided by the square root of the number of animals used before it is substituted in the formula), or by increasing the number of separate homogenates prepared. Obviously, the simplest way to reduce the per cent error would be to use several homogenates containing the pooled tissues of several animals.

It should be pointed out that the factor $\frac{(19.1)^2}{S}$ enters the formula only when one wishes to compare two different homogenates or groups of homogenates. It is omitted when comparisons within a single homogenate are to be considered.

The variation between the mitochondria counts for different homogenates (coefficient of variation, 19.1 per cent; Table II) raised the question whether this was caused by an inability to homogenize tissues reproducibly or whether

TABLE III

Showing mitochondria per cc. in homogenates of different concentrations. The mitochondria counts are expressed in terms of a 10 per cent homogenate. Columns I and II represent two experiments and A and B represent different operators counting from the same homogenate.

Homogenate Concentration in per cent	Mitochondria per cc. homogenate			
	I		II	
	A	B	A	B
5	9.6×10^9	8.4×10^9	11.0×10^9	8.0×10^9
10	8.4	8.8	9.5	8.2
10			10.9	9.1
10			10.6	9.4
15	9.2	8.6	10.4	10.1
20	8.8	9.8	12.0	8.9

these differences represented a true animal variation. Accordingly, two experiments were set up to test whether it was possible, firstly, to prepare replicate homogenates from the same pool of tissue and, secondly, to effect the same degree of cell rupture in homogenates of different tissue concentration. In the first experiment, the livers of ten C_3H mice were pooled, squeezed through a tissue press and the resulting mince was mixed thoroughly. From this homogeneous tissue mince, aliquots were removed, weighed and made to final tissue concentrations of 5, 10, 15 and 20 per cent by diluting with 0.25 M sucrose. The same weight of tissue was used for each homogenate with the result that the volumes of the homogenates were not the same. In the second experiment, homogenates (5, 10, 10, 10, 15, and 20 per cent) of constant volume and different tissue weight were prepared from a tissue mince obtained from the livers of 12 C_3H mice. In counting the mitochondria in all of these homogenates, the dilutions were graded so that approximately the same number of mitochondria were counted in each chamber. Each homogenate was counted by two operators.

The results of the experiments are summarized in Table III. Statistical analysis of the data was made both by comparing the differences between homogenates with the mean, assuming Poisson distribution, and with the differences between the chambers counted for each homogenate. The analysis showed that only in the case of count IIB was there a significant level of inhomogeneity, these counts having a coefficient of variation of 7.3 per cent. This variation obviously does not account for the 19.1 per cent coefficient of variation previously obtained with different homogenates. Therefore, the

homogenate differences recorded in Table II are clearly not due to errors in the technique of homogenization, but are largely an indication of animal variation. The data also show that it is possible to obtain the same degree of cell rupture in homogenates of tissue concentration varying from 5 to 20 per cent.

It is clear from Table III that the reproducibility of the counts made by one operator is very good and that counts made by one operator give a valid indication of variations in mitochondrial numbers in different material. The values obtained by the two operators were somewhat different and it would be unwise to compare counts from different material made by separate operators unless considerable effort were made to train each operator in the precise recognition of mitochondria in these preparations. Since the aim of the method is to compare values rather than to obtain absolute values, the reproducibility of the counts made by one operator is a far more important consideration than is a difference of this magnitude between the counts of separate operators.

DISCUSSION

The counting method described in this paper makes it possible to estimate with considerable accuracy the number of mitochondria in whole tissue homogenates or in isolated cell fractions. It would not be fair to say that the method is easy to perform since considerable patience and experience are required to achieve proper filling of the counting chamber and because preliminary study is needed in the recognition and identification of mitochondria. In spite of these technical difficulties, however, the results presented show that the method can attain a relatively high degree of accuracy and reproducibility after some experience has been acquired.

Setting the limits of accuracy of the mitochondria counts was conditioned largely by the use to which the count method was to be put, and the method is subject to considerable refinement if increased accuracy is desired. Increasing the accuracy of the method would involve the enumeration of a great many mitochondria by several previously trained operators counting from many samples of the same material, and when differences of a fairly large order of magnitude are to be measured it seems unnecessary to attempt such refinement. No attempt was made in these experiments to make an exhaustive analysis of the errors of measurement, but Magath *et al.* (9) have made a study of the errors involved in counting erythrocytes in a hemocytometer. These workers demonstrated that errors due to pipettes and chambers

were negligible when compared with the human error and that operator error could not be eliminated. Since it seems impossible to obtain accurate absolute counts of the number of mitochondria in a given preparation, the only decision to be made concerns the amount of error that can be tolerated when significant differences in mitochondrial numbers are to be demonstrated.

The ability to estimate the number of mitochondria in tissue homogenates will be of value in many types of research. It has already been used as a check on the purity of the fractions obtained by differential centrifugation of liver homogenates and on the recovery of mitochondria in the isolated fractions (7). Other areas in which the enumeration of mitochondria should prove valuable include the determination of the number of mitochondria in different tissues of the same and of different species and the establishment of the concentration of proteins and nucleotides within intact mitochondria. The latter information should be especially useful to the biochemist in elucidating and reconstructing the complex enzymatic systems known to be associated with mitochondria. Rough calculations of some of these concentrations have been made with the following results. Assuming an average mitochondrial diameter of 2 μ, the protein concentration within a mitochondrion corresponds to a 10 per cent protein solution if the entire mitochondrial nitrogen is considered to be protein, and to a 6 per cent protein solution on the basis of the mitochondrial proteins known to be soluble in nature (3, 4). Cytochrome c is a protein that has been localized in liver mitochondria (13, 14), and its calculated concentration within a mitochondrion is about 2×10^{-5} M. This value is of some interest because it is in the same range as the concentration (10^{-5} M) previously found necessary to saturate the succinoxidase system in rat liver homogenates (17). However, it is probable that the concentration of cytochrome c at the actual site of succinoxidase activity in the mitochondria is higher than the calculated value because it has been found that both cytochrome c and succinoxidase activity are localized in the insoluble or sedimentable fraction of the mitochondria (5). A calculation of the level of high energy phosphate within mitochondria is also of interest. Using the values reported by Kielley and Kielley for the labile phosphate content of mitochondria (8), an intramitochondrial concentration of 0.002 M is obtained. This value closely approaches the levels of adenosine-triphosphate usually added in current biochemical studies such as those on oxidative phosphorylation.

It is obvious that these calculations are valid only for a spherical mitochondrion 2 μ in diameter, and that a change in the diameter of the mito-

chondrion by as little as a factor of two would result in an eight-fold increase or decrease in the intramitochondrial concentrations. Since the size of mitochondria within intact cells and after release from cells is inversely related to the tonicity of the medium in which they are suspended (2, 6), the concentration of materials within the mitochondrion could be considerably different from those calculated above. These calculations, however, do give an indication of the order of magnitude of the concentrations of the substances in a mitochondrion.

In the study of carcinogenesis with azo dyes, Price *et al.* (10) have shown that feeding a highly carcinogenic dye causes a decrease in mitochondrial protein in the liver of rats, whereas feeding a non-carcinogenic azo dye leads to an increase in mitochondrial protein. Histological study of these livers indicated a corresponding change in mitochondrial numbers. A firmer estimate of these fluctuations could be obtained by counting the mitochondria in homogenates of these livers.

It has been known for several years that there is a great decrease in the amount of mitochondrial protein nitrogen in the liver tumors induced in rats by azo dyes (12) as well as in certain spontaneous mouse hepatomas (15). Recent studies in this laboratory have established that a given weight of mouse liver contains about 2.5 times as many mitochondria as an equivalent amount of hepatoma tissue. Experiments are now in progress in which correlation of mitochondrial counts with nuclear counts and cell volume estimates are being made in order to ascertain whether the differences in mitochondrial contents of liver and hepatoma actually represent differences in the number of mitochondria per cell.

Recent reports indicate that in certain hormonal disturbances, the activity of some liver enzymes known to be associated with mitochondria undergo marked variations. Thus, Tipton *et al.* (19) reported an increase in succinoxidase and cytochrome oxidase activity in the livers of rats fed thyroid powder, and Shipley *et al.* (18) report an increase in succinoxidase activity in the livers of diabetic rats. It would be of considerable interest to determine whether these enzymatic changes reflect an increase in the *number* of mitochondria or whether the *concentration* of these enzymes in the mitochondria is increased. Experiments by Dr. S. Wollman, of this Institute, on isolated liver mitochondria indicate that in the diabetic rat, the increase in succinoxidase activity is accompanied by an increase in mitochondrial nitrogen, while in the hyperthyroid rat the increased succinoxidase activity was not paralleled by an increase in mitochondrial material. It is planned to confirm these results by mitochondrial counts.

SUMMARY

A method has been developed for counting unfixed, unstained mitochondria directly in mouse liver homogenates by using a Petroff-Hausser bacteria counting chamber and the phase microscope. Aliquots of whole homogenate were suitably diluted in 0.125 M sucrose solution before counting.

An average of 8.7×10^{10} mitochondria was found to be present in each gram of fresh C_3H mouse liver. Statistical evaluation of mitochondrial counts from homogenates of 10 C_3H mouse livers showed that the mitochondria in the counting chamber followed Poisson distribution and that there was negligible variation in the counts between chambers. Some error was introduced when successive dilutions were made and counted from the same homogenate, but the greatest variance was found to occur between different homogenates. A formula is presented by means of which the error in mitochondria counts can be calculated when comparisons are to be made either within a single homogenate or between two or more homogenates.

Further experiments, in which homogenates of different concentration and of the same concentration were prepared from the same batches of tissue, showed that the difference between homogenates was due largely to animal variation and not to an inability to homogenize tissues reproducibly. These experiments also showed that it was possible to obtain the same degree of cell rupture in homogenates of tissue concentration varying from 5 to 20 per cent.

Some possible applications of the method are discussed.

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REPLACEMENT OF CARBON DIOXIDE BY FACTORS OBTAINED FROM LEBEDEV JUICE

D. T. O. WONG and S. J. AJL[1]

Department of Bacteriology and Immunology, Washington University School of Medicine, St. Louis, Missouri

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Lwoff and Monod (7, 8, 9) showed that under reduced CO_2 tensions the growth of *Escherichia coli* is decreased and that glutamic acid and other C_4 and C_5 dicarboxylic acids help restore growth. At very low CO_2 tensions, however, the C_4 and C_5 acids do not replace carbon dioxide, whereas yeast extracts and other complex materials do.

In a series of studies by Ajl and Werkman (1, 2, 3) data have been presented implicating a number of Krebs cycle intermediates as well as some of their metabolic precursors as substituents for the carbon dioxide requirement in the metabolism of *E. coli* and *Aerobacter aerogenes*. It was found that when the normal growth medium (0.4 per cent $(NH_4)_2SO_4$, 0.8 per cent KH_2PO_4, 0.8 per cent glucose and 20 per cent tap water) was aerated at the rate of 5 vol/min with CO_2-free air, *E. coli* did not grow appreciably. However, on the addition of either succinate, fumarate, malate, oxalacetate, α-ketoglutarate, aspartate or glutamate, growth did take place.

The ostensibly disparate results of the two laboratories stem from differences in the CO_2-tensions employed. The ability of any compound to replace the CO_2 requirement is a function of the absolute CO_2-tension in the growth medium. This was stated by Ajl and Werkman (1) in their original paper: "The rate of growth depends not only upon the addition of certain compounds but upon the rate of aeration . . ." Lwoff and Monod (9) state that in their experiments 20 times more CO_2-free air passed per unit time than in Ajl and Werkman's experiments and that these differences in methods may explain why Ajl and Werkman . . . "have failed to recognize the fact that CO_2 is not essential for the synthesis of C_4 and C_5 dicarboxylic acids only, but also for the formation of other heterocarboxylic metabolites," presumably present in yeast extracts.

[1] Present address: Department of Bacteriology, Army Medical Service Graduate School, Washington, D.C. (U.S.A.).

These interpretations are correct. Data will be presented in this communication which show that when the medium is aerated at the rate of 75 vol of CO_2-free air/min (instead of 5 vol/min as in the original work), none of the Krebs cycle intermediates, singly or in combination, will substitute for CO_2. Yeast extracts, Lebedev juices and other complex materials function. Further, the active substance (or substances) in Lebedev juice has been at least partially purified and some of the chemical and physical properties determined.

It is interesting to note that previous to both of these studies Scherp and Tuttle (10) reported that yeast extract will by-pass the supplemental CO_2-requirement of the meningococcus. Individual constituents presumably found in yeast extract were tested. Yeast nucleic acid was partially effective, adenine was not; however, guanine, uracil and cytosine individually partially replaced the CO_2 requirement. When all three were added together, they completely replaced the supplemental CO_2-requirement of this organism.

METHODS

Essentially, the growth experiments were performed as previously described (1). Some minor modifications, however, were introduced and they will be mentioned briefly. The most important modification was an increase in the rate of aeration with CO_2-free air to 75–100 vol/min. Normal air was freed from CO_2 by passing it through a series of alkali towers filled with glass beads containing 30 per cent NaOH. Second, the reaction tube contained 20 ml of basal medium (described in the introduction) instead of 100 ml as in the original work (1). This further facilitated the removal of CO_2 from the growth medium. Third, 1 per cent inoculum (by volume) of a 24 hr *E. coli* culture grown in the same basal medium was added aseptically. The bacteria were centrifuged down, washed several times with sterile distilled water, and aerated with CO_2-free air in acidphosphate buffer (pH 5.7) for several hours. The suspension was made up to standard turbidity and 1 per cent inoculum of these "CO_2-free" cells added. All experiments were conducted at 30° C. Growth was measured turbidimetrically on the Klett-Summerson photoelectric colorimeter with a 660 mμ light filter. All readings were made after 18 hrs incubation.

EXPERIMENTAL DATA

In Table I, the ability of a number of compounds to replace carbon dioxide under low and high aeration with CO_2-free air is compared qualitatively. The original findings of Ajl and Werkman (3) are confirmed, e.g., C_4 and C_5 Krebs cycle intermediates or their metabolic precursors *individually* substitute for carbon dioxide, whereas C_1, C_2, and C_3 compounds do not.

TABLE I

Ability of Krebs cycle intermediates or their metabolic precursors to replace carbon dioxide under low and high aeration with CO_2-free air.

Exp. No.	Conditions	C_1, C_2, and C_3 Compounds	Growth	C_4 Compounds	Growth	C_5 Compounds	Growth
A	Aeration with CO_2-free air, 5 vol/min. Total volume of growth medium = 100 ml	Pyruvate	7	Succinate	250	α-ketoglutarate	360
		Alanine	4	Fumarate	260	Glutamate	400
		Acetate	2	Malate	273		
		Glycollate	4	Oxalacetate	240		
		Formate	6	Aspartate	200		
B	Aeration with CO_2-free air 75 vol/min. Total volume of growth medium = 20 ml	Pyruvate	3	Succinate	7	α-ketoglutarate	4
		Alanine	5	Fumarate	6	Glutamate	7
				Malate	5		
				Oxalacetate	6		
				Aspartate	9		

All compounds were added in 0.2 mM quantities. Growth is indicated by turbidimetric readings on the photoelectric colorimeter. Control experiments, e.g., without CO_2-substituents, gave readings from 3–9. Incubation time 18 hrs. Temp. 30° C.

When the medium is aerated at the rate of 75–100 vol/min with CO_2-free air, this is no longer true. Under those conditions only complex materials such as yeast extract, peptone and Lebedev juice function as CO_2 substituents. These latter findings are identical with those reported by Lwoff and Monod (8).

A series of experiments were next performed in which various combinations of Krebs cycle intermediates, amino acids, vitamins, etc., were added in an attempt to replace carbon dioxide under high aeration. The results are the same as those obtained with single compounds under similar conditions, e.g., no replacement of CO_2 under low CO_2 tensions. (It is assumed, of course, that the absolute CO_2 tension in the medium at any given time is much lower when the reaction vessel is aerated at the rate of 75–100 vol/min than 5–7 vol/min.)

In view of the findings of Scherp and Tuttle (10) with the meningococcus, an attempt was made to replace the carbon dioxide requirement with several purine and pyrimidine derivatives. None of the compounds tested including creatinine, thymine, adenosine, guanosine, singly or in combination, replace CO_2 under low CO_2 tensions.

TABLE II

Purification of CO_2-Factor(s)

Total volume of Solution[1] ml	Treatment of Acidified Solution	Dry wgt/ml of Water Layer mg	Growth/ml of Water Layer
260	No treatment	67.0	185
250	Extraction; twice with ether	52.2	180
220	Extraction; twice with benzene	49.0	195
210	Extraction; twice with chloroform	23.8	180
200	50 grams of Dowex-1 (200–400 mesh)	22.0	190

The addition of 1 ml resulted in half maximum growth.

It follows, then, that the compounds which function as "complete" CO_2 substituents (replacing the gas at high and low aeration) are found in complex materials only and that these compounds are different from those we have thus far tested.

Purification of "Complete" CO_2 Substituents (CO_2-Factor) from Lebedev Juice.

Occurrence. It was previously stated that the CO_2-factor(s) occurs in a number of complex substances, most of which are ingredients of common microbiological media. Lebedev juice was chosen as the source for subsequent purification of the CO_2-factor(s).

Preparation. A low temperature dried, residual brewers yeast, obtained from Anheuser-Busch, was used as the starting material. Lebedev juice was prepared in the usual fashion by incubating a given quantity of dried yeast with three times its weight of distilled water at 37° C for 3 hrs. After this period, the cell debris was centrifuged and the supernatant liquid subjected to 48 hr dialysis. The dialysis was carried out with or without stirring at room temperature against distilled water. The dialysate replaced CO_2 under low and high aeration.

The dialysate was made alkaline (pH 10) and boiled down to one-tenth of its original volume. The solution was then adjusted to pH 7.0. A flocky precipitate separated out which was filtered off and discarded since it did not replace CO_2. The filtrate was subsequently subjected to the treatment shown in Table II. It is apparent that, although the various extractions

[1] The total solution was made to 260 ml.

TABLE III

Comparison between "complete" (alcohol fraction) and "incomplete" (succinate) carbon dioxide substituents.

Carbon Source in Growth Medium	CO_2 Substituents	Aeration with CO_2-free air vol/min.	Growth
Glucose	—	5	3
Glucose	Succinate[1]	5	180
—	Succinate	5	3
—	Succinate	5 (aeration with normal air)	200
Glucose	—	75	0
Glucose	Succinate	75	2–5
—	Succinate	75	0
—	Succinate	75 (aeration with normal air)	210
Glucose	Alcohol fraction[2]	75	200
—	Succinate + alcohol fraction	75	210
—	Alcohol fraction	75	25–40

The alcohol fraction was added in concentrations to give half maximum growth. Total volume of growth medium 20 ml. Growth is indicated by turbidimetric readings on the photoelectric colorimeter. Control experiments gave readings of 3–9.

removed a great deal of material, as evidenced by the reduction in the dry weight, the CO_2-factor(s) remained in the water layer. In each case all of the original activity was recovered. At the same time more than half of the total dry weight was removed by the various solvents.

The water layer, after treatment with Dowex, was evaporated to dryness under an infrared lamp. A thick, brownish, almost oily, gum-like material separated out. It was taken up in large volumes of absolute ethyl alcohol. Only a small fraction of the total material dissolved. The remainder no longer replaced CO_2. The alcohol layer was completely effective as a CO_2 substituent and the total dry weight necessary to give half maximum growth was 5.8 mg.

Properties. The CO_2-factor(s) in the alcohol layer is stable to autoclaving at 20 lbs pressure for 60 minutes at pH 2, 7, and 13.

The possibility that the activity might be due to traces of mineral constitu-

[1] 200 μM. [2] See text.

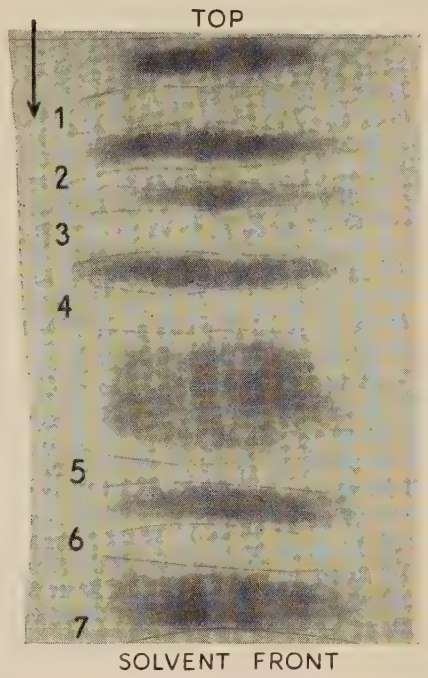

Fig. 1. Chromatogram of Alcohol Fraction.

ents was investigated by ashing the material and adding the neutralized ash solution aseptically to the autoclaved assay medium. The ash was completely devoid of CO_2-replacing activity.

Since the activity of the alcohol soluble fraction cannot be ascribed to its mineral constituents, the organic nature of the CO_2-factor(s) is self-evident. Steam distillation of the alcohol fraction adjusted to acid pH does not result in the volatilization of any activity. Other studies indicate that both aqueous and organic solvent solutions of the active substance may be best evaporated to dryness slowly at room temperature.

It is clear from the data in Table II that the dialysate of Lebedev juice functions as a "complete" CO_2-substituent. Several comparisons can now be made between the complete CO_2-substituent, as found in the alcohol fraction, and succinic acid, for example, which functions as a CO_2-substituent only at low aerations. The results are summarized in Table III. Under low aeration, glucose is necessary before succinate will function as a CO_2-substituent, although succinate alone can serve as a carbon source when the medium is aerated with normal air. The alcohol fraction, functioning as a complete CO_2-substituent will replace CO_2 when added alone. It was also

found in a separate experiment that the alcohol fraction by itself will support the growth of a number of microorganisms. This indicated that further purification of the alcohol fraction was mandatory.

Paper chromatography offered the best means for such purification. A quantity of the alcohol fraction was spread on a large sheet of Whatman No 1 filter paper and partitioned between tert-amyl alcohol, formate and water, according to the procedures of Lugg and Overell (6). The sheet was sprayed with 0.04 per cent bromcresol purple and photographed. The results are shown in Fig. 1. Apparently, there are at least 7 acidic compounds (these are the colorless bands on the chromatogram which actually show up as yellow with this dye) contained in the alcohol. We are now in the process of determining the nature of these compounds and their ability to function as CO_2-substituents. Preliminary experiments indicate that the second, third and fourth colorless bands (from the origin) replace carbon dioxide in the metabolism of *E. coli*.

DISCUSSION

It is clear from these and a great number of previous studies that organisms deprived of CO_2 will not develop even in media which contain good sources of carbon dioxide, notably glucose. Lwoff and Monod (7, 8, 9) and Ajl and Werkman (1, 2, 3) have raised the question whether the carbon dioxide requirement can be substituted with compounds arising from CO_2 fixation reactions. In a series of papers by Ajl and Werkman (1, 2, 3), it has been shown that when to the normal medium (containing glucose) are added C_4, C_5, or C_6 Krebs cycle intermediates, singly or in combination, the growth will be normal when the culture is aerated at the rate of 5–7 vol/min with CO_2-free air. Under the same conditions but in the absence of these compounds, the growth is retarded. In this paper, the same experiments were repeated at higher aerations, e.g., 75–100 volumes of CO_2-free air/min and under these conditions only complex organic materials such as yeast extracts and Lebedev juices, will function as CO_2-substituents.

Now, what do these results mean? It is first of all clear that both under low or high aeration these compounds do not function simply by supplying CO_2 to the medium. If this were the case pyruvate, for example, which is a good source of CO_2 for *E. coli*, should replace carbon dioxide. Further, glucose, which is always present in the growth medium is rapidly metabolized by this organism with the liberation of large quantities of CO_2, yet in the absence of carbon dioxide, the organism fails to develop, even in its presence. Now, as to the differences observed with low and high aeration. Assuming

that the absolute CO_2 tensions in the growth media at any given time are qualitatively, at least, different under low and fast aeration with CO_2-free air (e.g., approaching zero with 75 vol/min and considerably above zero with 5–7 vol/min) the following statements can be made.

Carbon dioxide is probably necessary for growth because it forms C_4, C_5 or C_6 compounds which are essential for normal respiration and metabolism. This may be assumed since compounds containing at least four carbons will replace carbon dioxide under low aeration. The orderly manner in which this replacement occurs has been described elsewhere (3). It does not follow, however, that every compound formed as a result of a CO_2 fixation reaction will replace carbon dioxide. For example, when *E. coli*, is incubated with $C^{14}O_2$ and pyruvate, the latter becomes labeled. Clearly, CO_2 has been exchanged. However, pyruvate will not replace CO_2. One must supply a compound with a minimum of four carbon atoms. This is not surprising. Glucose may break down to pyruvate in the absence of CO_2. Before growth can take place, C_4, C_5, and/or C_6 di- and tri-carboxylic acids must be formed because of their importance in respiration and metabolism in general. Under normal conditions, these compounds are presumably formed by CO_2 fixation reactions. However, they must be added when the CO_2 is even partially removed from the growth medium. The latter readily follows from chemical equilibria considerations. The equilibria of carbon dioxide fixation reactions are far to the side of decarboxylation, e.g., liberation of carbon dioxide. Thus, the problem faced by the cell is to shift the equilibrium as far as possible in the uphill direction. This task is simpler in the presence of large concentrations of CO_2. When the CO_2 tension is reduced, although not completely removed, CO_2 fixation becomes an impossible task and the cells fail to grow.

It does not follow, however, that C_4, C_5, and C_6 compounds are the only ones which the cell requires for multiplication and growth. This is evidenced from the data obtained at near zero CO_2-tensions. Under those conditions, other metabolites present in complex mixtures function, presumably also arising from CO_2 fixations and not yet completely identified. These are the precise conclusions arrived at by Lwoff and Monod (7, 8). It is entirely possible that, under low aeration and in the presence of an "incomplete" CO_2 substituent, a sufficient amount of CO_2 is present to allow the formation of some compound without which growth could not be initiated. When the CO_2 is completely removed, however, this is no longer possible and the incomplete CO_2 substituents no longer function as carbon dioxide replacement compounds.

Since we show in this paper that the complete CO_2 substituent is not one of the C_4, C_5 or C_6 Krebs cycle intermediates, or their metabolic precursors, it follows that the latter are only of secondary importance to the cell, and that they will only function in replacing CO_2 in the presence of minute amounts of the gas. The real problem is, therefore, to identify the complete CO_2 substituents, e.g., functioning at near zero or zero CO_2 tensions, and this is what we are in the process of doing.

The problem of CO_2 replacement is even more complex than that. We show in Table III that succinate can replace CO_2 under low aeration when glucose is present. In addition, succinate can serve as a carbon source for growth in the presence of CO_2. However, when the carbon dioxide is removed, succinate can no longer serve as a source of carbon. There is, therefore, an interreplacement of CO_2 between succinate and glucose, e.g., succinate replacing CO_2 in the presence of glucose and vice versa. Pyruvate, acetate and several other related substances will not substitute for glucose in the presence of succinate. Under high aeration, however, the alcohol fraction will replace CO_2 in the presence of succinate when the latter is serving as the carbon source. From the characteristics of the alcohol fraction we are reasonably certain that it does not contain carbohydrate. Therefore, glucose could not function in the alcohol fraction as a substituent for CO_2 when succinate is the sole carbon source.

The problem of CO_2 replacement, particularly under extreme low CO_2 tensions, remains open to further investigation. It is hoped that many of the problems pointed out above will be solved by analysis of the alcohol fraction and by identification of the various compounds shown on the chromatogram.

One other problem need be emphasized in connection with this work on CO_2 replacement; e.g., the findings with one organism do not necessarily apply to other bacteria. For example, Gerhardt and Wilson (5) report that none of the Krebs cycle intermediates or a number of the amino acids, including glutamate and aspartate, will replace the supplemental requirements of carbon dioxide in freshly isolated strains of *Brucella abortus*. Farghaly (4), on the other hand, working with luminous bacteria, found that although Krebs cycle intermediates do not function as CO_2 substitutes, a number of amino acids are capable of overcoming the CO_2 requirement of these bacteria. The extent of replacement was a function of the rate of aeration. The findings are exactly analogous to those reported in this paper with amino acids. Farghaly suggested that the amino acids may function by affecting the metabolism of the organism so as to lead to the production

of CO_2 which is necessary for the synthesis of other unidentified compounds. It is likely that these compounds are contained in the alcohol fraction which are in the process of being purified and identified.

SUMMARY

C_4, C_5, and C_6 Krebs cycle intermediates or their metabolic precursors replace carbon dioxide in the metabolism of *E. coli* only when the medium is aerated at the rate of 5–7 volumes of CO_2-free air/min. Under identical conditions the normal medium containing glucose gives very little growth. When the medium is aerated at the rate of 75 vol/min none of the Krebs cycle intermediates have an effect. Only complex materials such as yeast extracts, peptone or Lebedev juices, will replace carbon dioxide. One such complex source, e.g., Lebedev juice, was partially purified and some of the chemical and physical properties determined.

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POSTMORTEM MITOTIC ACTIVITY OF SPERMATOGONIA AND SPERMATOCYTES IN THE ALBINO RAT

E. C. ROOSEN-RUNGE

Department of Anatomy, University of Louisville School of Medicine, Louisville, Kentucky

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In 1950 Bullough (1) reported that cells of the epidermis and intestinal mucosa of mice did not enter mitosis after the death of the animal, but that divisions begun before death were usually completed. However, cells of mammary carcinoma and spindle cell sarcoma entered mitosis for at least 2 hours after death. Three to 4 hours postmortem only a few mitotic figures were observed in normal or tumor tissue.

The present investigation began when it was accidentally found that in the seminiferous tubules of the rat mitotic figures do not disappear for many hours after death of the animal. Quantitative methods for the determination of proliferative rates of spermatogonia and spermatocytes had been developed previously (7, 8). It was thought to be of interest to use these methods in an investigation of the mitotic behavior of these cells under conditions of relative anaerobiosis.

MATERIALS AND METHODS

The rats used were of Sprague-Dawley strain. All were 4 ½ to 6 months old and weighed between 225 and 320 grams. The animals were stunned by a blow on the head and immediately afterwards the heart was severed. In nearly all cases one testis was used as a control and fixed at the time of death. The other testis was left *in situ* and removed 1 to 16 hours afterwards. The killings were done between 8 and 9 A.M. with the exception of the 16-hour cases, which were killed at 2 P.M. In about half the cases the testes were fixed by injection of the fixing fluid (Stieve's solution) into the testicular artery under the tunica albuginea after the removal of the organ from the body. This technique is particularly successful with testes 3 to 10 hours after death which are otherwise not easily fixed without distortions to the tissues.

The testes were embedded in paraffin, cut at 12 μ, and stained with Heidenhain's Iron-Hematoxylin. Mitoses were counted according to the method described in a previous paper (8). In each case at least 2 areas of about 150 sections of tubules were counted in each testis. The areas were always taken from 2 regions several millimeters apart.

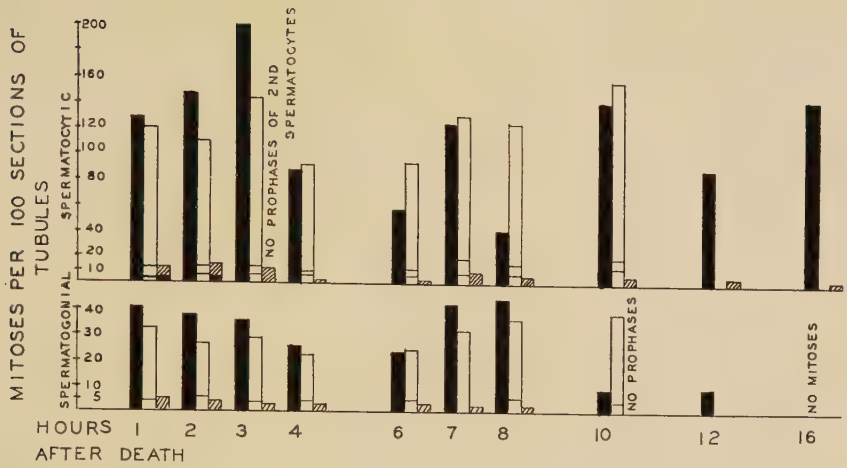


Fig. 1. Postmortem mitotic activity of spermatogonia, primary and secondary spermatocytes. Black columns: frequencies of mitoses postmortem. White columns: controls (from the other testis, fixed at death). Third columns: frequencies of prophases postmortem. In the upper part of the graph the prophase column is subdivided into primary (hatched) and secondary (black) spermatocyte prophases. The lines in the white column in the lower part indicate prophase frequencies in the controls, in the upper part the prophase frequencies of the controls have been subdivided into primary (above) and secondary (below) spermatocyte prophases.

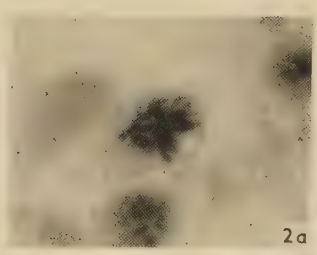
The 1 and 2 hour values are averages of 3 cases each, 2 kept at room temperature (about 28° C), 1 at 37° C. The 8 hour values are averages from 2 cases, both at 37° C. All other values are from single cases kept at body temperature.

Only those mitotic figures were counted which could be identified beyond doubt. The definitions of phases were entirely arbitrary, based on practicability. A prophase was regarded as beginning when a spireme was clearly visible in the spermatogonia or when the nuclear membrane was in the act of disappearing in the spermatocytes. Its end was determined by the appearance of the typical metaphase plate. Telophase was defined as starting with the first sign of constriction of the cell body and ending when the cells were completely separated.

Experiment with colchicine: One rat received a subcutaneous injection of 3.5 mg colchicine per 1000 gm body weight ½ hour before death. At death the right testis was removed. The body was kept at 38° C, and 3 ½ hours after death the left testis was removed.

RESULTS

Fig. 1 summarizes the quantitative findings of this investigation. It can be seen that the behavior of spermatogonia, primary and secondary spermatocytes is very different. The three cell kinds will, therefore, be described separately with special emphasis on those observations which are not shown in the graph.



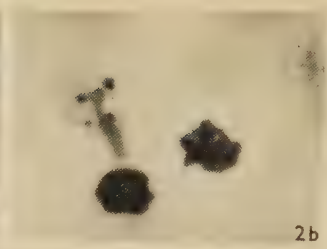


Fig. 2. Spermatogonial mitoses 16 hours postmortem, kept at about 28° C. × 1 430. Sections 12 μ thick. a) Metaphase of type A cell. Chromosomes show only slight clumping. b) pyknotic figures of type B cells from the same preparation. The cytoplasm of these cells has become almost unstainable.

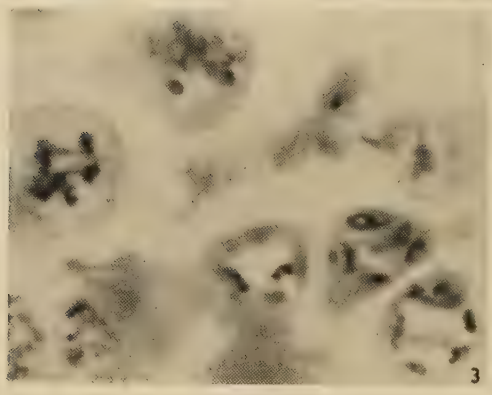


Fig. 3. Prophases of primary spermatocytes 12 hours postmortem, kept at 38° C. In the lower right, tetrads, in the upper left, late prophase figures with beginning spindle formation (less conspicuous than normal) in one of the cells. × 1 430. Sections 12 μ thick.

Spermatogonia. Mitosis in these cells appears entirely normal as long as 8 hours postmortem. After that the mitotic figures become more and more clumped, and after 16 hours at 37° C only a few figures can be identified (Fig. 2b). After 16 hours at room temperature metaphase plates of type A spermatogonia are still found in fair condition (Fig. 2a) although the metaphases of type B cells are definitely abnormal. For the first 3 hours after death the number of spermatogonial mitoses is high when compared with the controls. From 3 to 6 hours it is within the normal, but high values are once more recorded after 7 and 8 hours. From then on normal mitoses become rare, and none are found after 16 hours. The prophase counts are normal until 8 hours postmortem. After that time no more spermatogonial prophases are seen. Telophases which, in the control testes and in testes up to 4 hours after death vary from 4 per cent to 14 per cent of all mitoses, increased to 24 per cent and 28 per cent 6 and 8 hours postmortem. Thereafter the counts decrease rapidly. After 10 hours a few of the telophase nuclei are observed in pairs in undivided cells. After the sixth hour the resting nuclei of the type B spermatogonia become progressively changed. The chromatin, which is normally arranged in clumps at the periphery of the nucleus, stains more and more heavily and becomes more sharply defined.

The resting nuclei of type A cells maintain normal appearance for a longer time. Even after 16 hours some of them are not appreciably changed.

Primary spermatocytes. These cells show mitoses in normal numbers and near normal condition even 16 hours postmortem (Fig. 3). While their divisions would normally constitute about $\frac{1}{3}$ of all spermatocyte divisions they increase to $\frac{2}{3}$ or more a few hours after death, so that their values in Figure 1 indicate very high numbers of primary and low numbers of secondary spermatocytes. After the sixth hour the spindle fibers become progressively less stainable, and cannot be found at all after 12 hours. There is also a slight tendency to clumping. The prophase counts are normal for the first 3 hours after death. At 4 and 6 hours they are far below the controls. After this time they increase again, but remain definitely below the level of the first 3 hours. The low prophase values at 4 and 6 hours can, perhaps, be correlated with the low number of mitotic figures of primary spermatocytes found 2 and 4 hours later. The small early primary spermatocytes show abnormalities after 10 hours. Their nuclei become progressively pyknotic. After 12 hours they are the most heavily stained structures in the tubules. The diplotene stages appear well preserved until at least 10 hours postmortem, and tetrad figures can be recognized easily after 16 hours.[1]

Secondary spermatocytes. These cells show completely normal mitotic behavior only for the first 2 hours after death. Prophases disappear almost completely after this time. Absolute and relative numbers of mitoses decrease steadily. After 16 hours the resting nuclei of these cells are thoroughly abnormal. Their chromatin has become granular instead of showing the normal reticular arrangement.

General observations. While the germinal epithelium is, as a whole, well preserved for many hours after death certain changes do occur within and outside of the seminiferous tubules. One of the first is the appearance in the lumen of the tubules of precipitations in globular masses which stain with hematoxyline. Another is a progressive loss of cohesion between the cells of the germinal epithelium accompanied by a retraction of the Sertoli cells and a rounding of their nuclei. From the seventh hour on conspicuous cellular changes are more frequently encountered. The interstitial cells appear shrunken, their nuclei either ballooned or pyknotic. The spermatid

[1] A similar observation was made in a human case. The testes of a man who came to autopsy 2 $\frac{1}{2}$ days after drowning, and whose body had been in the water for a considerable time, showed well preserved primary spermatocytes with only slightly abnormal mitotic figures, although the interstitial tissues were in very poor condition and filled with bacteria.

nuclei begin to change progressively and the heads of the spermatozoa appear bent and stain only in part.[1]

After 16 hours at 38° C rod-like bacteria 3½ to 4½ μ in length are found frequently, singly and in chains, in the interstitial tissue. They are extracellular. None are seen in the tubules. At this time the testes are very soft and have a strong odor of putrefaction. After 12 hours at 38° C prolonged search reveals a few isolated bacteria in the interstitial tissue. Before this time none were seen.

At 1 and 2 hours postmortem the mitotic counts of animals left cooling slowly to room temperature and those kept at 37–38° C show no appreciable difference. After 16 hours the testis is definitely better preserved at a lower temperature. The picture corresponds approximately to that of a testis kept for 8 hours at near body temperature.

The effect of colchicine. The results of the colchicine experiment and its controls are summarized in Table I. The high number of spermatogonial mitoses found consisted of about 80 per cent metaphase figures in the clumped condition typical for the effect of colchicine. The number of prophases was not significantly decreased. The spermatocytes were not appreciably changed by the drug, and their metaphases were not arrested. This behavior corresponds to that found in rats that lived to the time of fixation (7).

DISCUSSION

The main point of discussion is the question whether cells found postmortem in any of the phases of mitosis have truly survived until the time of fixation, or whether they have become "fixed by death" in a cytological *rigor mortis*, as it were.

Common experience does not affirm the unchanged state of mitoses after death. Tissues which are not fixed soon after death usually show a conspicuous lack of mitotic figures. Bullough (1) showed that mitoses in the skin, the intestine and in lymph nodules were usually completed within 2 to 3 hours after death of the animal. In the skin after 3 hours only a few telophases remained. In the intestine a static condition did, indeed, appear to exist with the exception of the prophases which were completed and disappeared after the first hour. Bullough speculated that the rapid action

[1] It appears to the author that controlled autolysis may well prove to be a valuable technique in the hands of experienced cytologists as it has already proved to be with some bacteriologists (4). In nuclei of spermatids and in the heads of spermatozoa some definite stainable structures appear 12 hours after death and later.

TABLE I

Comparison between frequencies of spermatogonial and spermatocytic mitoses in colchicine treated and control animals.

	Number of mitoses per 100 sections of tubules		Prophases per 100 sections of tubules
	spermato-gonial	spermato-cytic	
1. Control testis of 2. Fixed at death	29	107	2.9
2. 4 hours after colchicine. 3 ½ hours after death	62	120	2.0
3. 4 hours after colchicine (7)	102	135	not counted
4. 4 hours after death	25	88	2.5

of digestive enzymes on the cells might be responsible for this phenomenon which he did not find in other cells of the body.

Three major items may be presented as evidence for prolonged survival and actual progression of mitoses after death of the animal.

1) The accumulation of typical arrested spermatogonial metaphases found 3 ½ hours after death in an animal injected with colchicine ½ hour before death can only be explained by the fact that a considerable number of cells entered prophase and proceeded to metaphase after death. The effect of colchicine does not become apparent until 30 to 40 minutes after the injection (7) and the control testis removed at the time of death shows no arrested metaphases and no increase in the number of spermatogonial mitoses (Table I). Three and a half hours later the number of spermatogonial mitoses has approximately doubled. This increase in numbers of spermatogonial mitoses for the first hours postmortem in conjunction with a lack of increase in the number of prophases (Fig. 1) makes it very probable that mitoses begin and proceed after death, although they do so at a slower pace than during life.

2) Prophases disappear in the secondary spermatocytes after the second hour, in the spermatogonia after the eighth hour. In the primary spermatocytes they still can be found after 16 hours, although they have decreased in number compared with the first 3 hours after death. There is no evidence that prophases which disappeared became necrotic and died. These prophases must, therefore, have progressed to the metaphase state, and we may assume that the cells were alive when this transformation occurred in the secondary spermatocytes during the third hour, in the spermatogonia during the seventh and eighth hour, and in the primary spermatocytes even

later. As a corollary to this deduction one feels inclined to regard the presence of prophase as in itself a sign of life, although rigorous proof for this is at present missing.

3) If the mitotic state after death were entirely static the numbers of mitoses counted at varying intervals should not exceed the range of variations of the controls. Actually, they show far greater fluctuation. The meaning of these fluctuations is at present obscure but the fact that the numbers of mitoses fluctuate as they do can also be taken for an indication that living phenomena are present. It should be pointed out that the gradual disappearance of spermatogonial mitoses after the eighth hour cannot be completely accounted for by necrosis of mitotic figures. The number of abnormal and necrotic mitoses is never as high as the normal number of spermatogonial mitoses. The conclusion is, therefore, that at least some cells have actually completed mitoses as late as 8 hours after death.

To the evidence presented the general impression is added that the appearance of mitotic figures and of the cytoplasm of these cells is normal for many hours postmortem.

The assumption is safe that the germ cells are living after death with very little if any free oxygen available, and the conclusion is inescapable that germ cells show a considerable ability to exist under relatively anaerobic conditions. Further investigations will have to determine the source of energy under these conditions. It is, however, extremely interesting that histochemical glycogen determinations show an abundant glycogen supply in the spermatogonia, in the small growing spermatocytes and in the Sertoli cells, but not in the fully matured primary nor in the secondary spermatocytes (5). It has not yet been determined how long the glycogen supply of the testis lasts after death.

When Pütter in 1905 (6) wrote the first thorough account of the survival of protozoa under strictly controlled anaerobic conditions he postulated that anaerobic metabolism is the general and primitive way in which living matter obtains its energy supply, and that aerobic metabolism is a more efficient, specialized form of respiration. It was later shown that embryonic cells of birds and mammals appear to be more resistant to lack of oxygen than do the adult tissues (for instance 2, 3 and 10). The ability of the germ cells to live anaerobically would fit well into this general scheme. It may also be remembered that germ cells have been shown by Warburg to produce relatively great amounts of lactic acid anaerobically and aerobically (9).

SUMMARY

1. In the rat spermatogonial mitoses can be found in all phases, and in normal or slightly above normal numbers for 8 hours after death of the animal. Prophases disappear after this time. Sixteen hours after death no normal spermatogonial mitoses are seen. The type A spermatogonia show normal mitotic figures for a longer time than the type B cells.

2. Mitoses of the primary spermatocytes are found in normal numbers up to 16 hours postmortem. At this time only a few prophases are present.

3. Secondary spermatocytes ordinarily do not enter prophase after the second hour postmortem.

4. It is concluded that spermatogonia and primary spermatocytes can survive and show mitotic activity for many hours after death under definitely anaerobic conditions. They may, therefore, be added to the list of cells known to survive under conditions of relative or absolute oxygen deprivation; i.e. certain protozoa, the cells of certain invertebrates, certain embryonic cells and certain malignant tumor cells.

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SOME OBSERVATIONS ON THE ACTION OF TRYPSIN ON SEA URCHIN EGGS

A. BOHUS JENSEN

The Biological Institute of the Carlsberg Foundation, Copenhagen, Denmark and The Institute for Cancer Research and the Lankenau Hospital Research Institute, Philadelphia, Pa., U. S. A.

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Evidence accumulated during recent years shows that trypsin interferes with the fertilization process in different ways. According to these reports (21, 29) complete inhibition of fertilization results if a sufficiently high concentration of the enzyme is used. High percentages of cross fertilization due to the presence of trypsin were obtained with sea urchin eggs (11, 12). Furthermore, Morgan (18) found that *Ascidian* eggs which are normally self-sterile could be self-fertilized after treatment by a trypsin solution. It may be mentioned here also that several cases of artificial activation of unfertilized eggs by trypsin have been reported. In view of the fact that neither egg nor sperm cells are visibly damaged by trypsin, it seemed worthwhile to gain further insight in the specific manner in which the enzyme interferes with the act of fertilization. For this reason the following investigations were carried out, mainly dealing with the inhibitory effect of the enzyme on the fertilization of sea urchin eggs.

TECHNIQUE AND MATERIAL

The experiments reported here were carried out at the Zoological Stations of Kristineberg, Sweden, and Naples, Italy, with egg and sperm material from the following species of sea urchins: *Psammechinus miliaris* (S and Z forms) at Kristineberg, and *Psammechinus microtuberculatus*, *Paracentrotus lividus* and *Sphaerechinus granularis* at Naples. Eggs from a single female were used for each experiment in order to provide as uniform material as possible for the comparison. All experiments were performed at room temperature (13–20° C for the Neapolitan material and 20–24° C for the Swedish material), with eggs which had been washed in normal sea water before use. In all the experiments eggs with intact jelly coats were used. The crystalline trypsin preparations were obtained from Armour Laboratories, Chicago and from K. Linderstrøm-Lang, Carlsberg Laboratory, Copenhagen. The trypsin

TABLE I

Unfertilized eggs of *Psammechinus miliaris*, Z form were kept in a trypsin solution for 1/2 hour, thereafter inseminated in the presence of trypsin.

Conc. of trypsin in per cent	Experiment		Control	
	total number of eggs	percentage of cleaving eggs	total number of eggs	percentage of cleaving eggs
0.1	100	0	106	94.3
	100	0	107	93.5
	100	0	212	94.3
	104	3.8	103	97.1
0.01	205	97.6	203	98.5
	205	97.6	212	94.3
	306	65.4	207	96.6
	221	90.5	203	98.5
	210	95.2	208	96.2
	200	100	203	98.5
	217	69.1	215	93.0

solutions were dialysed before use against normal sea water for 24 hours in a refrigerator. The concentrations given in the following text include the inorganic salts contained in the original preparations.

EXPERIMENTS

The present investigation has amply confirmed that no fertilization takes place when insemination is carried out in sea water containing a sufficiently high concentration of trypsin. The results of two series of experiments carried out with the Swedish sea urchins at two different levels of concentration of trypsin are summarized in Table I.

Some experiments performed with the two Neapolitan sea urchin species gave similar results. Four batches of unfertilized eggs of *Sphaerechinus granularis* were kept for 1/2 hour in a 0.1 per cent trypsin solution and thereafter inseminated in the presence of trypsin. 76 to 95 per cent of the eggs showed no sign of fertilization while in the control samples kept in normal sea water 96–100 per cent of the eggs showed membrane elevation and cleavage. Similar experiments with 3 batches of unfertilized eggs of *Paracentrotus lividus* kept in 0.1 per cent trypsin solution 7–15 minutes; 95–100 per cent of the eggs showed no sign of fertilization while in the controls 90–100 per cent of the eggs were fertilized.

Table II

Unfertilized eggs of *Psammechinus miliaris* Z form were kept in a 0.1 per cent solution of trypsin in sea water for 1/2 hour.

Insemination in the trypsin solution		Insemination after washing of the eggs in normal sea water	
total number of eggs	percentage of cleaving eggs	total number of eggs	percentage of cleaving eggs
100	0	226	11.5
100	0	181	17.1
100	0	227	12.0
100	0	287	23.3
100	0	219	8.7
200	0	214	32.2
200	0	278	28.1
206	2.9	242	71.7
200	0	237	15.6
200	0	256	21.9
202	1.0	313	63.9

It could be shown, however, that if the eggs were washed after the trypsin treatment and insemination was carried out in normal sea water a varying number of the eggs regained their fertilizability. The results of these experiments carried out on the Swedish material are summarized in Table II.

The spermatozoa were always found to be highly motile in the presence of trypsin, giving no visible signs of injury. It was furthermore observed that the spermatozoa accumulated in great numbers in the jelly coats of eggs of *Paracentrotus lividus* which had been treated with 0.1 per cent trypsin for 10 minutes and inseminated in the presence of the enzyme, in spite of the fact that at least 95 per cent of the eggs could not be fertilized. In experiments with three different batches of eggs of *Sphaerechinus granularis*, a number of cases of polyspermic fertilization were observed after insemination of eggs which had been treated with trypsin 1/2–2 hours (conc. 0.01–0.05 per cent). In these experiments the eggs were washed in normal sea water before insemination was carried out. While in the controls nearly all the eggs had cleaved into 4–8 cells, 25–50 per cent of the eggs in the experimental samples had divided into a larger number of cells (up to 20–25 cells) at the same time and were developing in a more or less irregular manner, which is a good indication that polyspermic fertilization had taken

place (7, 9). The conditions under which polyspermic fertilization takes place after treatment of the eggs with the enzyme are not well defined, however, since in several experiments under similar circumstances no polyspermy was observed.

Numerous attempts were made to induce activation of unfertilized sea urchin eggs by treatment with trypsin solutions. Although the results of these experiments have been wholly negative, it seemed of interest to communicate a few details about them since several authors have observed nuclear activation and imperfect cleavage after treatment of the eggs with trypsin (cf. discussion). Egg and sperm material from all the species mentioned above were used. The eggs were treated in trypsin solutions, the concentrations of which varied from 0.001–0.5 per cent. In some of the experiments the eggs were simply kept under observation in the trypsin solutions for several hours; in other experiments they were kept for shorter or longer periods in the trypsin solutions before being transferred to normal sea water for further observations.

A few observations were made on the effect of trypsin on the hyaline layer. It is assumed that the material from which the hyaline layer is built up is located inside the plasma membrane until the formation of the hyaline layer after fertilization takes place and that it is thereby undoubtedly protected against the action of the enzyme. Egg material of *Sphaerechinus granularis* kept in 0.1–0.01 per cent solution of trypsin for 15–20 minutes thereafter washed and inseminated in normal sea water, developed a hyaline layer on the surface of the egg. In the same experiment carried out with eggs of *Paracentrotus lividus* kept for 10 minutes in 0.1 per cent solution, washed and inseminated in normal sea water, a well developed hyaline layer was also observed.

It was observed, however, that the hyaline layer was formed in the presence of trypsin and withstood its action. Eggs of *Sphaerechinus* were placed in a trypsin solution (0.1–0.02 per cent). After a few minutes they were inseminated in the presence of trypsin. A hyaline layer developed and was observed in an intact state 1–2 hours after the insemination. Likewise eggs of *Paracentrotus lividus* were placed in a trypsin solution (0.1–0.01 per cent). The eggs were inseminated in the presence of trypsin, a hyaline layer developed and was found in an undamaged state 4 1/2 hours later. Very few eggs were fertilizable in 0.1 per cent trypsin as pointed out above. In these experiments where cleaving eggs were kept in trypsin solution no separation of the cleavage cells was observed. This further indicates that the hyaline layer was intact (cf. discussion).

DISCUSSION

Considering the amount of information now available about the effect of trypsin on eggs of sea urchins and a few other organisms, it seems worthwhile to attempt a critical evaluation of these data in some detail in order to clear the ground for further investigations.

It has previously been reported that pure trypsin exerts an inhibitory effect on the fertilization of eggs of *Psammechinus miliaris* (21, 29) and *Pomatoceros triqueter* (15). Moore (17) mentions that fertilization of the eggs of *Strongylocentrotus purpuratus* and *S. droebachiensis* is inhibited by pure trypsin, without giving further data. Furthermore it was noted (3) that an impure preparation of ficin blocked the fertilization of eggs of *Ps. mil.*, Z form, presumably due to the proteolytic enzyme.

It is a well established fact that trypsin (and probably other proteolytic enzymes as well) does not digest living sea urchin eggs (20, 23) and it is obvious also from the present investigation that the eggs can be kept for many hours in the enzyme solution without visible signs of damage. The evidence produced in this paper that a certain percentage of trypsin inhibited eggs can be made fertilizable again further supports this statement. Under the experimental circumstances of the present investigation no effect of trypsin on the segmentation of the fertilized egg was observed (cf. also 20), and there is at present no indication that trypsin penetrates through the plasma membrane and gets into the interior of the eggs (19). Concerning the effect of trypsin on the hyaline layer see below. Moore recently stated (17) that unfertilized eggs of *Dendraster excentricus* left in trypsin solution 2–3 hours showed irregularities of form ("amoeboid form") which he ascribes to the damaging effect of trypsin. It must be kept in mind, however, that an elevation of the fertilization membrane had taken place at the same time due to the presence of trypsin, and the present writer is of the opinion that these aberrant forms may be due to an imperfect activation which eventually leads to disintegration of the cells. These stages resemble those observed when unfertilized eggs have been activated by treatment with impure preparations of ficin (3) or trypsin (2).

In other words, we have at present no indication that the block of fertilization by trypsin is due to a general damage of the eggs by the enzyme. As the inhibiting effect of trypsin is demonstrated by inseminating the eggs in the presence of trypsin, it is of importance to decide whether the enzyme in any way interferes with the vitality of the sperm cell. It does not seem to be the case, however. It has already been mentioned that the spermatozoa show lively

movements even in high concentrations of trypsin and it has furthermore been shown (30) that the presence of pure trypsin prolongs the lifespan of spermatozoa, presumably not due to the fact that trypsin is a proteolytic enzyme. Spikes (25), however, has found that the fertilizing power of sperm cells (from *Strongylocentrotus purpuratus* and *Lytechinus pictus*) decreased rather rapidly when kept in a solution of cryst. chymotrypsin (concentration not indicated) although the motility of the spermatozoa was unimpaired. It is a question whether this is due to differences in the activity of the two enzymes or whether it is due to other differences in the experimental conditions. It was shown by Tyler and Fox (28) that the sperm agglutinating component (gynogamone 2, fertilizin) of egg water from *Strongylocentrotus purpuratus* is fairly rapidly destroyed by trypsin. Furthermore it has been found (22) that androgamone 2 (from *Echinocardium cordatum* sperm) was rapidly inactivated by trypsin. Since, however, the exact role of these two gamones in the normal fertilization process is not quite understood (27) at present, it cannot yet be decided whether the interference of trypsin with the gynogamone 2 or androgamone 2 has anything to do with the inhibiting effect of trypsin on the fertilization process, although it seems likely. The fact that a certain percentage of the eggs regain their fertilizability after washing may be explained by assuming that new gynogamone 2 is produced thereby making the fertilization possible. It seems at present most reasonable to assume that the inhibition is due to the proteolytic activity of the enzyme molecule although it may be of interest in future experimental work to consider whether other properties of the molecule may be responsible for the observed effect.

Since there is no indication of any real damage to the cell by trypsin, we may assume that essential structural elements of the plasma membrane are not attacked by trypsin. Protein material is generally assumed to play an important role in the structure of the plasma membrane (6, 10), but as is well known, protein structures are not necessarily subject to a breakdown by trypsin even under *in vitro* conditions (4, 1). Furthermore it is often assumed (10) that it is in the form of lipoprotein complexes that the proteins form an essential part of the plasma membrane and there is reason to believe that they are to some extent protected against action of a proteolytic enzyme in that way (26). It may be mentioned here that the present author, using a rather pure trypsin preparation (Novo, Copenhagen), was unable in a series of unpublished experiments to confirm Herrmann's and Fischer's claim (8) that trypsin causes hemolysis *in vitro*.

Very little is known at present about the effect of trypsin on the mechanism

which prevents more than one sperm cell from entering the egg. However, irregularities of cleavage which have occasionally been observed (29, 23) after insemination of sea urchin eggs in the presence of trypsin, may be due to the entrance of several spermatozoa into the egg. Furthermore several polyspermic eggs were observed after treatment of eggs of *Pomatoceros triqueter* with pure trypsin (15). Since the number of observations still is small, it seems premature to attempt any explanation, but it is of interest to recall that a variety of means which are supposed to cause a slight injury to the eggs makes possible polyspermic fertilization (14).

The evidence in literature of the activating influence of trypsin on unfertilized sea urchin eggs is somewhat conflicting and at present seems to indicate that there are specific differences in the susceptibility of the eggs toward the enzyme. Previous workers have found that impure preparations of trypsin (2, 13, 24) and ficin (3) cause an activation of unfertilized eggs of a number of organisms. Runnström (21) found that a cryst. trypsin preparation induced an elevation of the fertilization membrane followed by a nuclear activation of unfertilized *Echinocardium cordatum* eggs, and Moore (17) recently described the activation of *Dendraster excentricus* eggs after treatment with a pure trypsin preparation. An elevation of the fertilization membrane took place, but no further development occurred (cf. also 15). The present writer (2), however, found no effect whatsoever after treatment of *Psammechinus microtuberculatus* eggs with pure trypsin while an impure preparation readily activated the eggs. This clearly emphasizes the need for the use of pure enzyme preparations in studies of this kind.

In what way the enzyme is interfering with the surface structure of the egg in the cases where development is initiated is not known, but it must be kept in mind that the vitelline membrane, the precursor of the fertilization membrane is situated outside the plasma membrane (5) and is therefore directly exposed to the action of the enzyme (it is rapidly broken down by trypsin (21)). The mere fact that the enzyme at a suitable concentration produces an elevation of the fertilization membrane need not be an indication that trypsin molecules have passed through the plasma membrane, the boundary of the living cytoplasm, and entered into the interior of the egg as assumed by Moore (17). The interference of the enzyme with the structure of the vitelline membrane itself or its attachment to the surface of the egg may lead to the initiation of development.

A few observations have been published previously on the effect of proteolytic enzymes on the hyaline membrane. Using a crystalline trypsin preparation, Runnström (21) found that in the presence of the enzyme the cleavage

cells (*Psammechinus miliaris*) "were loosely connected or completely dissociated" (cf. also 22), and Moore (16) observed that when a sufficiently high concentration of trypsin was used the hyaline membrane was dissolved in the case of egg material of *Strongylocentrotus purpuratus* (no information is given about the quality of the trypsin preparation). Northrop (20), however, obtained normal development of fertilized *Arbacia* eggs in the presence of pure ficin and trypsin and it has further been confirmed (3) that eggs of *Psammechinus miliaris* developed a hyaline layer in the presence of an impure ficin preparation and that the formed layer was resistant to the action of the enzyme and no falling apart of the cleavage cells was observed. It seems most reasonable at present to assume that the failure of trypsin to influence the state of the hyaline layer in Northrop's and my investigations is due to a too low activity of the enzyme under the experimental conditions.

SUMMARY

1) Observations made by previous authors that trypsin inhibits fertilization have been confirmed and extended. It was found that a certain number of trypsin inhibited eggs can be made fertilizable again by washing in sea water.

2) Cases of polyspermic fertilization after treatment of eggs with trypsin were observed.

3) Activation of unfertilized eggs by means of trypsin was not obtained in a large series of experiments.

4) The hyaline layer was found to be resistant towards the action of trypsin under the experimental circumstances described.

5) It is pointed out that there is no evidence at present that trypsin is affecting the viability of eggs and sperm cells.

6) It is suggested that trypsin inhibits the fertilization process by inactivating gynogamone 2 (and perhaps also androgamone 2).

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X-RAY DIFFRACTION STUDIES ON THE EFFECT OF TEMPERATURE ON THE STRUCTURE OF MYELIN IN THE SCIATIC NERVE OF THE FROG

J. ELKES[1] and J. B. FINEAN

Department of Pharmacology, University of Birmingham and Davy Faraday Laboratory, The Royal Institution, London, England

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Observations on the X-ray diffraction pattern of irrigated frog sciatic nerve, and a study of the effect of drying upon this pattern, have been reported (8, 4, 5). Present experiments are concerned with some effects of temperature on whole nerve, and on total nerve lipids.

EXPERIMENTAL

Materials. Frog sciatic nerve was used throughout. Total nerve lipid extract was obtained by refluxing the tissue with alcohol followed by ether, and evaporating the pooled extract to dryness at reduced pressure.

Source of radiation, and diffraction cameras. These have been previously described (4, 5). In all exposures made at long specimen-to-film distances, hydrogen was used to reduce air scatter (5).

Observation cells. Three cells were employed. The first of these (Fig. 1) was used to cover a range of temperature from 20° C to −90° C. The freshly dissected sciatic nerve preparation, with spinal column attached, was mounted in the irrigation chamber, through which frog Ringer solution was circulated continuously. The nerve, which had been ligatured at its point of entry into the gastrocnemius muscle, was passed through an aperture in the bottom of the irrigation chamber, and was allowed to hang freely inside the brass cylinder. A small weight (0.6 g) attached to the nerve served to steady it during the experiment. The cylinder was fitted with cellophane windows for passage of X-rays. The system was cooled by liquid air which was introduced into the bottom of the cylinder through a side tube at a controlled rate. As the liquid air evaporated, it produced a stream of cold air which served to cool the specimen before escaping through an aperture at the top of the cell. A heating coil around the brass cylinder was used to keep the cellophane windows free from frost at temperatures down to about −5° C. Frosting at lower temperatures was prevented by directing a jet of air against the windows. The temperature was measured by means of a thermocouple hooked around the nerve at the point of incidence of the X-ray beam.

In some experiments, short segments of fresh nerve, ligatured at each end, were

[1] Present address: Department of Experimental Psychiatry, Medical School, Birmingham 15.

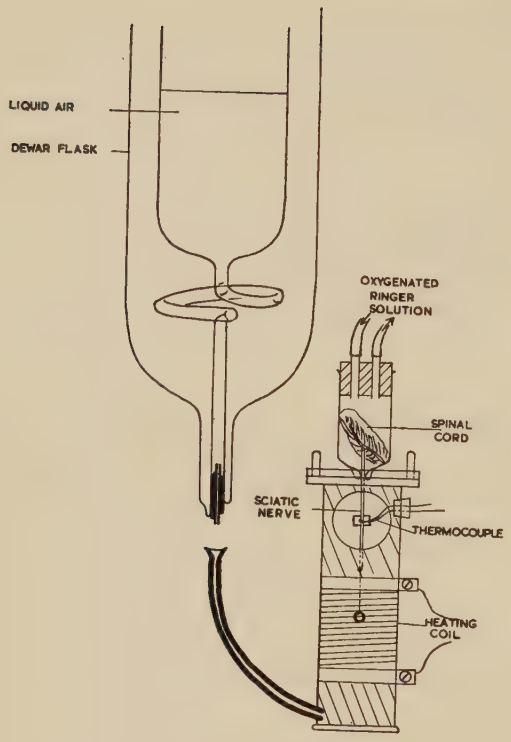

Fig. 1. Diagram of low temperature cell (cooled by liquid air).

attached to a metal frame, and a stream of liquid air directed against them. Cooling to —180° C was thus quickly achieved, and the diffraction pattern was recorded at this temperature.

The second cell (Fig. 2) used over a range of +80° C to —40° C, was a modification of one previously described (5), and made provision for the simultaneous recording of the X-ray diffraction patterns and nerve conductivity. A sciatic nerve with the gastrocnemius muscle attached was used in these experiments. The muscle was mounted in the manner indicated in Fig. 2, and the muscle response recorded by means of an optical lever. A stream of air (which had been pre-cooled by passing through a copper coil immersed either in a solid CO_2-alcohol mixture or in liquid air) was blown into compartment C, and cooled the nerve segment in this compartment. The temperature of the specimen was controlled by the rate of air flow, regulated by a needle valve. A thin-walled glass or cellulose acetate capillary tube was slipped over the segment of nerve in compartment C to prevent drying. When a glass tube was used, a short segment of nerve at the bottom of the compartment was left unprotected so that direct contact with the thermocouple could be made. An interval of 5 to 10 minutes was allowed for equilibration of temperature. As most of the segment of nerve in compartment C was exposed to the X-ray beam, temperature readings at the bottom of the cell could be taken as representing mean values within

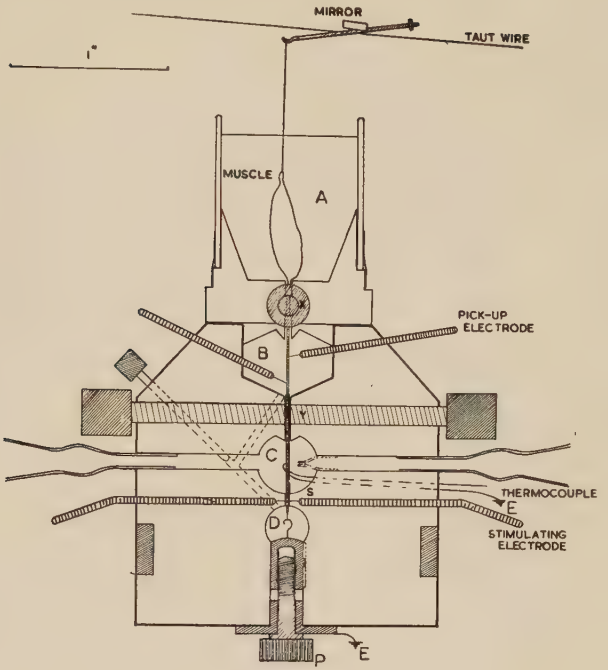

Fig. 2. Diagram of irrigation cell.

the limits of the experiment. In experiments in which nerve conductivity was studied simultaneously, however, this arrangement left some doubt as to the exact temperature at which changes of activity took place; a large (4 mm) segment of nerve was affected by the temperature changes, and the variation of the temperature along the specimen was not known. This difficulty was to some extent obviated by using a cellulose acetate capillary tube, and by inserting the thermocouple through a hole in the side of the tube. The specimen was then heated or cooled by a fine jet of air which impinged on the tube at the same height as, but opposite to the thermocouple. In this way only a small section of the nerve was affected, and the temperature was measured at that point. The X-ray diffraction patterns were not recorded in these experiments.

To study the ranges above room temperature, warm air was blown into compartment C. In this cell, temperatures could be maintained to within $\pm\frac{1}{2}^\circ$ C for periods of up to two hours.

A third cell served to cover a range up to 300° C. This consisted of a brass block, electrically heated, in which the temperature was controlled through an electric contact thermometer which was in thermal contact with the brass block. The specimen was placed in a compartment in the block, and a thermocouple touching the specimen, served to record the temperature. The exposed surfaces of the cell were insulated with a layer of asbestos. Temperatures could be maintained to within a degree for periods up to three hours. It was not found necessary to provide windows for the specimen compartment.

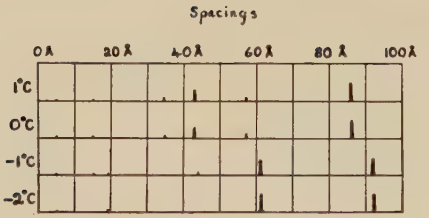

Fig. 3. Summary of changes in diffraction pattern observed over the temperature range +1° C to —2° C.

METHODS

Experiments with fresh nerve were carried out over a range of temperatures from +80° C to —90° C. In many experiments, series of exposures were made using the same specimen, the temperature being raised or lowered between exposures. Between temperatures of +8° C and —5° C, and 58° C and 63° C, the diffraction patterns were examined at intervals of ½° C to 1° C; other ranges were covered at intervals varying from 2° C to 5° C.

The effects of speed of cooling, and of freezing a second time after thawing, were also examined. In studying the diffraction patterns obtained after thawing, the length of time during which the nerve had remained frozen, and the temperature at which it was maintained, were taken into consideration.

In all experiments, the specimens were finally dried to constant weight, and the diffraction patterns compared with that of normal dried nerve.

The effects of temperature on the diffraction pattern of normal dried nerve, nerve dried after freezing and thawing, and total lipid extract of nerve, were also studied. A temperature range of —20° C to +200° C was covered, the region between —20° C and +60° C at intervals of 5° C to 10° C, and higher regions at intervals of 20° C to 30° C. The diffraction patterns of lipid-in-water emulsions were examined at temperatures of —20° C to 100° C, with particular reference to +2° C to —2° C.

RESULTS

In the figures which follow, most of the dimensions given for diffraction spacings represent mean values derived from a number of experiments carried out under slightly varying conditions. The maximum variations observed in these experiments are stated.

Some diffraction bands, particularly the very weak ones, were not observed in a sufficient number of patterns for satisfactory mean values to be obtained. In such cases only approximate spacings are given.

a) Fresh nerve. Cooling. 85 exposures were made at temperatures below 20° C. Of these, 50 were taken between +8° C and —5° C. In all exposures, the X-ray beam was perpendicular to the fibre direction. Fig. 3 summarizes the findings, and typical diffraction patterns are shown in Fig. 4. There were

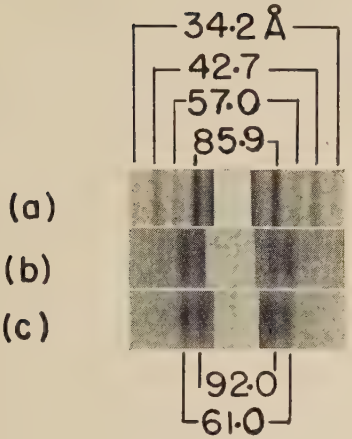

Fig. 4.

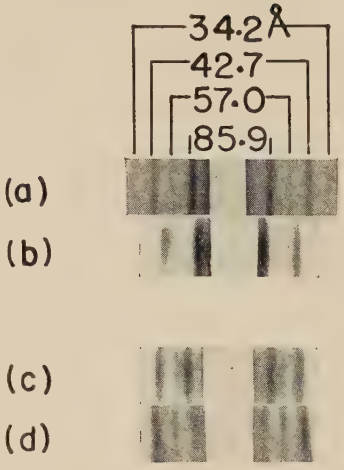

Fig. 5.

Fig. 4. Examples of diffraction patterns observed during cooling. (a) At 20° C. (b) At —2° C. (c) At —40° C.

Fig. 5. Effects of thawing and drying on the diffraction pattern of fresh nerve. (a) Fresh nerve pattern. (b) Pattern after freezing and thawing. (c) Normal dried nerve pattern. (d) Pattern of nerve, dried after freezing and thawing.

no variations in diffraction pattern which could be attributed to the rate of cooling, the pattern depending solely on the actual temperature at the time of exposure.

The first changes in the nerve pattern were observed near the freezing point of Ringer solution, as indicated by the appearance of an ice diffraction pattern in the short spacing photographs. At this point (i.e. between 0° C and —2° C) the normal fresh nerve pattern was replaced by one showing intense diffraction bands at 61.0 ± 1.5 Å and 92.0 ± 2.5 Å (Fig. 4b). Some patterns showed an additional faint band at about 46 Å. The 15.0 ± 0.2 Å band of fresh nerve disappeared on freezing, and was replaced by a band at 19.65 ± 0.5 Å which showed less equatorial orientation. The 4.7 ± 0.1 Å band was difficult to examine because of interference by diffractions from ice and from the cellophane window, but it did not appear to have undergone any marked change. As the temperature was lowered further (to —90° C) there was a steady decrease in intensity of the 92.0 ± 2.5 Å band in relation to the one seen at 61.0 ± 1.5 Å. At —180° C, the diffraction pattern consisted of a broad, diffuse band at about 63 Å, and a very faint band at about 90 Å.

Thawing and drying. In eight experiments the fresh nerve specimen was frozen for 15 minutes (i.e. the time usually required for recording the

diffraction pattern), allowed to thaw, and then immersed in Ringer solution for at least thirty minutes. Material treated in this way showed two well-defined diffraction bands at 97.4 ±6.0 Å and 47.8 ±3.0 Å. The variation of spacings in these patterns was greater than the estimated maximum experimental error, and appeared to be related to the duration of freezing, and the temperature maintained during this period. In general, it seemed that the longer the period of freezing and the lower the temperature, the higher the spacing seen on thawing and immersing in Ringer solution. Further faint bands were observed at about 15 Å and 20 Å, and occasionally at about 40 Å. In some experiments in which the flow of Ringer solution down the specimen was very slow, the first pattern to be recorded after thawing resembled that of fresh nerve. However, after further treatment with Ringer solution, the pattern changed to the one just described.

When dried, all specimens gave a pattern which consisted of an intense band at 42.8 ±1.0 Å, and weaker bands at 59.6 ±1.5 Å and 92.4 ±3.5 Å (Fig. 5d). In addition, some patterns showed a very faint band at about 34 Å. The relative intensities of the 92.4 ±3.5 Å, and 59.6 ±1.5 Å bands appeared to vary according to the conditions of the experiment. The longer the duration of freezing, and the lower the temperature maintained, the weaker the 92.4 ± 3.5 Å band.

Freeze-drying. Freeze-dried nerve furnished diffraction patterns similar to those obtained from normal dried nerve material (Fig. 5c).

Refreezing. In some of the experiments, the specimen was allowed to thaw for a few minutes, and then frozen again. In such cases, there was a further decrease in intensity of the 92.0 ±2.5 Å band relative to the one at 61.0 ±1.5 Å. Repeated freezing and thawing resulted in the almost complete disappearance of the 92.0 ±2.5 Å band. The intensity of the 92.4 ±3.5 Å band in the pattern of the dried specimen showed a corresponding decrease.

Heating. 15 exposures were made between 40° C and 70° C, 11 of these being taken between 50° C and 60° C. The normal fresh nerve pattern persisted up to about 55° C, but above this temperature the bands began to decrease in intensity and to become diffuse. Between 58° C and 61° C there was a definite change in diffraction pattern. The new pattern (Fig. 6b) consisted of a moderately intense but somewhat diffuse band at 75.5 ±4.0 Å, and a very broad and weak band at 41.0 ±1.2 Å. At higher temperatures the general intensity of this pattern decreased.

Cooling (following initial heating) and drying. When the specimen was cooled to room temperature after initial heating to 65° C for fifteen minutes, the 75.5 ±4.0 band showed an increase in dimension and definition, whilst

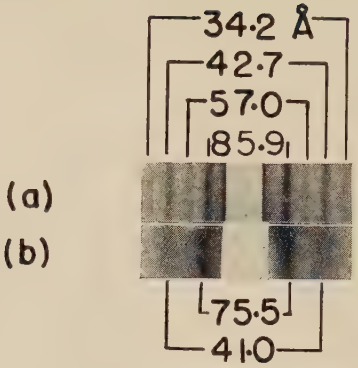

Fig. 6. Effect of heating on the diffraction pattern of fresh nerve. (a) At 20° C. (b) At 62° C.

the diffuse band at 41.0 ±1.2 Å showed a decrease in intensity, and was eventually replaced by a sharp band at about 54 Å. In six experiments the nerve was heated in the irrigation cell, removed after cooling, and left in Ringer solution at 20° C for an hour. Specimens thus treated gave diffraction patterns which showed well-defined and intense bands at about 96 Å and 48 Å (Fig. 7) the former being the more intense. In some cases, very faint bands could also be seen at about 59 Å and 35 Å.

In other experiments, lengths of nerve, ligatured at either end, were heated in Ringer solution, and then left to cool before the diffraction patterns were examined. When the bath of Ringer solution was maintained at a temperature of 60° C for ten minutes, and then allowed to cool slowly, the final diffraction pattern was similar to that obtained in the previous experiment. In all, seven such specimens were examined. When subsequently dried, each gave a diffraction pattern which showed a moderately intense band at 44.6 ±1.5 Å, a less intense band either at about 56.7 Å or at about 60 Å, and a higher spacing which varied considerably both in magnitude (80 to 105 Å), and intensity. These variations again appeared to bear some relation to the extent of previous heating and immersion in Ringer solution.

Effect of temperature on impulse conduction. This was found to vary a good deal, and to depend upon the rate of change of temperature, as well as the actual temperature level. Thus, on cooling rapidly, conduction was blocked at temperatures as high as 6 or 8° C, though on lowering the temperature slowly, conduction could often be maintained until just above the freezing point of Ringer solution. No muscle response was ever observed after ice had formed in the specimen (as detected by the ice diffraction pattern in the short spacing photograph).

In cases where blocking was seen at temperatures above the freezing point,

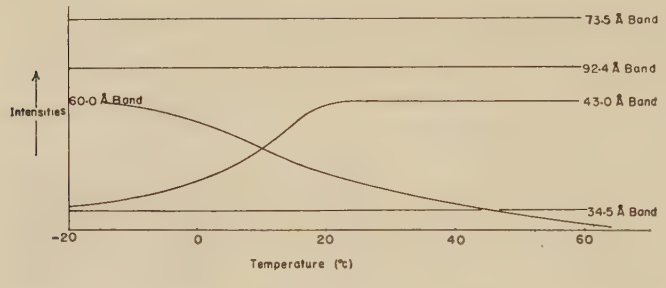

Fig. 7. Diagrammatic representation of the variations of intensities with temperature of diffraction bands in the patterns of normal dried nerve, total lipid extract, and nerve, dried after initial freezing and thawing.

activity was usually restored almost instantaneously when cooling was discontinued. Once freezing had taken place, however, no recovery was seen upon thawing.

Somewhat similar results were observed on heating. Blocking was seen at temperatures as low as 35° C, though recovery could usually be brought about by cooling. At higher temperatures, recovery was delayed, though it must be stressed that in our experiments, no consistent blocking temperature could be determined. The findings are in general agreement with those of previous workers (1, 2, 3, 6, 7, 9).

b) *Dried nerve and dried lipid extract.* Three series of experiments, involving a total of 30 exposures were carried out on dried nerve. A similar number of observations was made on total lipid extract.

The changes in diffraction pattern with temperature of normal dried nerve and dry total lipid extract showed marked similarities. The 73.5 ± 1.5 Å and 143 ± 3.0 Å bands of the dried nerve pattern appeared to be relatively unaffected over the whole range of temperatures covered (Fig. 7). The 34.5 ± 1.0 Å band, which was observed in both dried nerve and lipid extract patterns, showed little change below a temperature of 150° C to 170° C. At this temperature the band became diffuse and weak. Because of their relative stability these three bands at 73.5 ± 1.5 Å, 143 ± 3.0 Å and 34.5 ± 1.0 Å, formed useful standards for comparison. Marked variations in intensities were encountered in the 43.0 ± 1.0 Å and 60.0 ± 1.0 Å bands. Here again there was good agreement between dried nerve and total lipid extract. The approximate changes in intensities of the diffraction bands from dried nerve, total lipid extract, and nerve dried after initial freezing and thawing, are summarised in Fig. 7. Temperatures were maintained only whilst the X-ray diffraction pattern was being recorded (usually about 15 mins.). Diffraction patterns of dried nerve and total lipid extract at various temperatures are shown in Fig. 8.

At 20° C, the 43.0 ± 1.0 Å band was much more intense than the 60.0 ± 1.0 Å

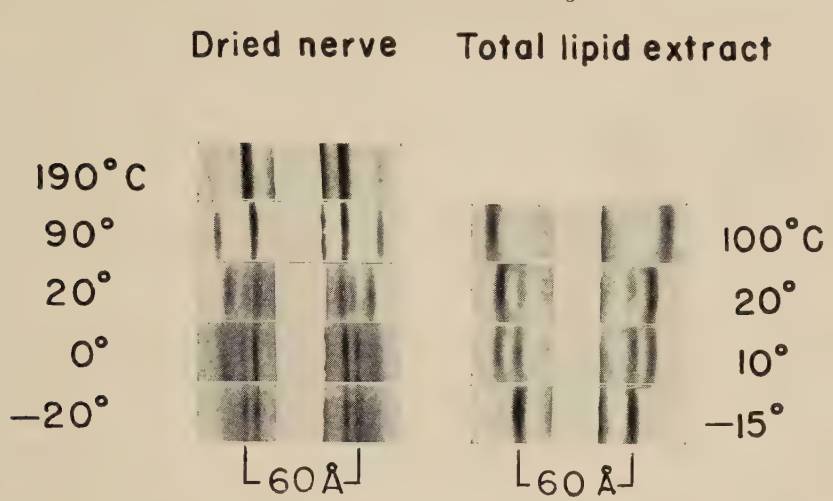

Fig. 8. Examples of diffraction patterns obtained from normal dried nerve and total lipid extract of nerve at different temperatures.

band, but on cooling, the intensity of the former decreased while that of the latter increased. Eventually the 60.0 ± 1.0 Å band became the more intense of the two. The intensity of the 43.0 ± 1.0 Å band appeared to be at a maximum at about 20° C, and remained relatively unchanged up to about 150° C. At this temperature the band became diffuse and weak. The intensity of the 60.0 ± 1.0 Å band decreased with rise in temperature, and the band was not seen at temperatures above 60° C to 70° C.

All spacings showed a gradual contraction as the temperature was raised from 20° C to 150° C. The effect was most marked in the case of the 43.0 ± 1.0 Å spacing which was reduced to about 39 Å before the band finally became diffuse at about 150° C.

The effects of temperature on the pattern of nerve dried after initial freezing and thawing (Fig. 5 d) were very similar to those observed in normal dried nerve and total lipid extract. The 92.4 ± 3.5 Å spacing was not reduced by more than 1 to 2 Å on heating to about 200° C.

c) Lipid emulsion. Total lipid extract was emulsified with an equal amount of Ringer solution. The diffraction pattern of the freshly prepared emulsion was always diffuse, but the definition of the bands improved when the preparation was allowed to stand for a day or two. At a temperature of 20° C, the diffraction patterns showed a fairly intense band which varied from 75 Å to 95 Å in different preparations. In addition, there was a less intense band at about half of this dimension. On cooling the emulsion, the only

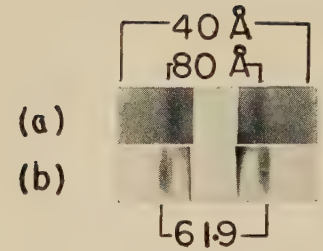

Fig. 9. Effect of freezing on the diffraction pattern of a 50 per cent emulsion of total lipid extract in Ringer solution. (a) At 20° C. (b) At —5° C.

marked change in diffraction pattern was observed at the freezing point. Here, the pattern changed abruptly to one which showed only a broad and intense band at 61.9 ± 2.0 Å (Fig. 9). The value of this spacing appeared to be independent of the initial spacings of the emulsion at room temperature.

On heating the emulsion, there appeared to be a gradual contraction of spacings (to the extent of about 10 Å at 100° C). At this temperature, the stronger band was seen to be split into two distinct spacings, one approximately equal to that observed at room temperature and the other corresponding to the spacings noted in the pattern of the frozen emulsion. Both bands were quite sharp, and the changes were reversible.

DISCUSSION

The diffraction patterns of fresh nerve, normal dried nerve and of nerve studied during intermediate stages of drying, have been previously discussed (8, 4, 5). The conclusions will be used in a consideration of the above results.

When fresh nerve was cooled in Ringer solution, the most marked change in diffraction pattern was observed in the region of the freezing point (about —1° C). Owing to slight fluctuations of temperature within the irrigation cell, and possible temperature variations over the comparatively large segment of nerve examined, it was impossible to establish whether or not these changes in diffraction pattern took place abruptly at the freezing point. The experiments showed that between 0° C and —2° C the normal fresh nerve pattern was replaced by a pattern characterised by well-defined diffraction bands at 19.65 ± 0.5 Å, 61.0 ± 1.5 Å, and 92.0 ± 2.5 Å.

In interpreting the diffraction pattern of frozen nerve, two possibilities must be borne in mind. The diffraction bands may all be related to a single fundamental structural unit, or they may be derived from several different systems existing as separate phases.

The three main spacings could be identified with second, third and

ninth orders of a hypothetical fundamental unit of about 180 Å. This might mean that the changes observed on freezing reflect an expansion of the 171 Å unit (proposed in the interpretation of the fresh nerve pattern (8, 4, 5)) to about 180 Å. Such expansion may be due to the conversion of water into ice. If the expansion is not restricted in any direction, and there is water throughout the length of the 171 Å unit of fresh nerve, the increase in length on freezing would be about 3 per cent or 5 to 6 Å. With expansion restricted in a direction at right angles to the long axis of the unit (e.g. by van der Waals cohesive forces between hydrocarbon chains), the increase in the length of the unit due to freezing might be considerably more than 3 per cent. If the patterns of normal and frozen nerve are to be related in this way, the explanation for the marked differences in relative intensities of orders may lie in a possible rearrangement of diffracting material within the unit, which could be brought about by an uneven expansion of the structure, changing the positions of diffracting groups relative to the length of the unit.

A comparison of the diffraction pattern of frozen nerve and that observed on freezing a 50 per cent lipid-in-Ringer emulsion suggests an alternative explanation of the changes in diffraction pattern which accompany the freezing of fresh nerve. The frozen emulsion gives a diffraction pattern which shows only one intense band at 61.9 ± 2.0 Å, whereas frozen nerve shows a 61.0 ± 1.5 Å band and an additional intense band at 92.0 ± 2.5 Å. The 61.0 ± 1.5 Å band of frozen nerve may reflect the existence of a free lipid component of similar constitution to that of the total lipid extract, but the additional band at 92.0 ± 2.5 Å would indicate that myelin contains some structure which is not reproduced in the lipid extract.

A similar conclusion is reached when the diffraction pattern of normal dried nerve and that of dried total lipid extract are compared (Fig. 10). The three lower spacings (34.5 ± 1.0 Å, 43.0 ± 1.0 Å, and 60.0 ± 1.0 Å) of dried nerve are reproduced by the total lipid extract, but not the 73.5 ± 1.5 Å and 143 ± 3.0 Å bands. The stability of the latter spacings at (73.5 ± 1.5 Å and 143.5 ± 3.0 Å) distinguishes them from the shorter lipid spacings. They are, however, affected by lipid solvents. It is possible that these longer spacings reflect a complex or complexes which involve lipid material, either alone or in combination with some non-lipid component, and that such complexes are broken down during extraction with lipid solvents.

There is a marked similarity between the diffraction pattern observed at the end of stage I of the drying of fresh nerve (5), and that of frozen nerve. In both cases the pattern appears to indicate the separation of a labile lipid component from the complex. However, the changes in diffraction pattern

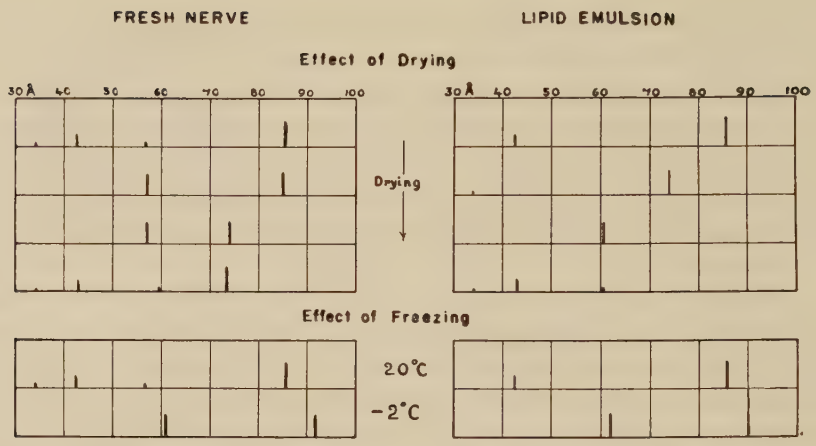

Fig. 10. Diagram comparing the effects of drying and of freezing on the diffraction patterns of fresh nerve and a 50 per cent emulsion of total lipid extract in Ringer solution.

observed during the first stages of drying are reversible, whereas those associated with freezing are not. In this connection, it is perhaps significant that freeze-dried preparations give a diffraction pattern which is identical with that of normal dried nerve, whereas a frozen nerve which is allowed to thaw before drying gives a diffraction pattern which differs from that of normal dried nerve in that it shows a band at 92.4 ± 3.5 Å rather than at 73.5 ± 1.5 Å. There is a corresponding difference between the higher spacings (i.e. in the region of 140 to 160 Å). It is thus possible that a significant change in structure takes place on thawing.

The fact that the pattern changes accompanying the freezing of an emulsion of total lipid extract are reversible, suggests that it is the complex component (not reproduced in the total lipid extract) rather than the labile lipids of myelin which is affected irreversibly by freezing and thawing.

The temperature changes in the diffraction pattern may be due to a change in a protein component, or a lipid component, or both. The presence of a protein component in nerve myelin has been suggested (8, 4, 5), but there is no positive X-ray diffraction evidence for its existence. Any diffraction from a small quantity of protein in myelin would probably be masked by diffraction from the larger amounts of protein in other components (e.g. axon, neurilemma, connective tissue). Nevertheless, it is of interest that the irreversible changes in diffraction pattern of myelin come within the range of protein denaturation. The changes in organisation of lipid might be expected to be reversible. Investigations of the nerve lipids are being continued.

SUMMARY

1. The effects of temperature on the X-ray diffraction patterns of fresh nerve, normal dried nerve, total lipid extract, and emulsified total nerve lipids, have been studied.

2. Maximal changes in diffraction pattern of fresh nerve were noted between 0° C and —2° C, and between 58° C and 61° C. The patterns of dried nerve and total lipid extract showed wide variations in the relative intensities of the 43.0 ±1.0 Å, and 60.0 ±1.0 Å bands. Except for a slight decrease in spacings, the other bands remained relatively unaffected over most of the range covered (—20° C to 200° C). Total nerve lipids emulsified in Ringer solution, showed a marked change in diffraction pattern at the freezing point.

3. A comparison of the changes in diffraction patterns of fresh nerve and emulsified total lipid extract, and those of dried nerve and dried total lipid extract, suggests the presence in myelin of a lipid or lipoprotein complex not present in total nerve lipid extract. The changes in the structure of this complex with temperature appear irreversible. The irreversible changes occur within the range of protein denaturation.

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EFFECTS OF SOLVENTS ON THE STRUCTURE OF MYELIN IN THE SCIATIC NERVE OF THE FROG

J. ELKES[1] and J. B. FINEAN

Department of Pharmacology, University of Birmingham, and Davy Faraday Laboratory, The Royal Institution, London, England

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The effects of drying, and of temperature changes on the structure of myelin in the sciatic nerve of the frog have been previously reported (2, 3). The purpose of this communication is to present some data on the effects of lipid solvents on the structure of myelin as studied by X-ray diffraction.

EXPERIMENTAL

Materials. Frog sciatic nerve was used in all experiments.

Apparatus. The source of X-rays, diffraction cameras, and observation cells have been described (1, 2, 3).

Methods. 1. *Exposure to solvent vapours.* Fresh nerve was mounted in the irrigation cell (2, 3), and a very slow stream of air, bubbling through alcohol, ether, or acetone, was passed into chamber C (2, 3). A series of diffraction patterns was recorded during this treatment. Immediately after recording the last diffraction pattern, the specimen was either dried by blowing a stream of air into the chamber, or re-wetted with Ringer solution. Action potentials were recorded during these experiments.

2. *Exposure to Ringer solution containing lipid solvents.* Fresh nerve was placed in Ringer solution, to which varying proportions of alcohol or acetone had been added. The diffraction patterns were obtained after varying periods, the specimens being bathed in the solvent-Ringer mixture during exposures. Specimens were dried at various stages of treatment, and the diffraction patterns of the dried material compared with those of normal dried nerve.

3. *Exposure to pure solvents.* (a) Extraction of lipid from both fresh and dried nerve was carried out at low temperature, reduced pressure, or under normal atmospheric conditions. The diffraction patterns, both of the residual tissue, and of dried lipid extract, were recorded.

(b) The effects of refluxing solvents over dried nerve were studied in a special distillation cell (2). The solvents used were alcohol, acetone, and ether.

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[1] Present address: Department of Experimental Psychiatry, Medical School, Birmingham 15.

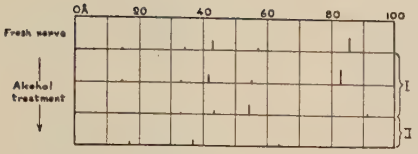

Fig. 1.

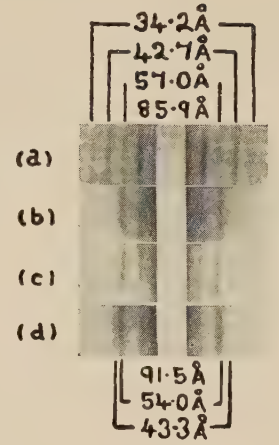

Fig. 2.

Fig. 1. Summary of changes in diffraction pattern observed during treatment of fresh nerve with alcohol vapour.
Fig. 2. Examples of diffraction patterns obtained during treatment of fresh nerve with alcohol vapour. (a) Fresh nerve pattern. (b), (c) and (d) Patterns obtained during treatment with alcohol vapour.

RESULTS

In the figures which follow, most of the dimensions given for diffraction spacings represent mean values derived from a number of experiments. The maximum variation observed in these experiments is stated. In cases where the number of observations made was insufficient only the approximate values of the spacings are given.

1) Solvent vapours. Ten series of experiments (each involving four to ten exposures) were carried out using alcohol, four series using acetone, and five using ether.

When a slow stream of air was bubbled through alcohol and passed into chamber C of the irrigation cell, the action potentials of the fresh nerve were blocked within a few minutes, and a series of changes in diffraction pattern was observed. A summary of these changes is given in Fig. 1, and examples of the diffraction patterns in Fig. 2.

The changes in diffraction pattern can be conveniently described as occurring in two stages. During stage I, all the spacings of the normal fresh nerve pattern showed a slight contraction, and at the same time the intensity of the band at 57.0 ± 1.5 Å (seen in fresh nerve pattern) began to increase. This increase in intensity, together with a further reduction in spacing,

continued after the other spacings had reached a minimum and had started to increase again. In four experiments minimum spacings of 41.5 ± 0.3 Å and 82.7 ± 0.6 Å were recorded, but by the time the intermediate band (initially at 57.0 ± 1.5 Å) had reached its maximum intensity, the main spacings of the pattern were 43.3 ± 1.0 Å, 54.0 ± 1.7 Å, and 91.5 ± 4.0 Å. Some patterns showed additional faint bands at about 35 Å and 17 Å. Spacings above 100 Å were not recorded.

In stage II, the pattern appeared to change rather abruptly to one which showed a fairly sharp and intense band at 37.0 ± 1.0 Å, and fainter bands at 63.4 ± 1.5 Å and 17.0 ± 0.1 Å. The intensity of the 17.0 ± 0.1 Å band was greater than in stage I. These spacings were calculated from nine diffraction patterns, and in every case the nerve specimen appeared to be quite soft and moist after the exposure. When treatment with alcohol vapour was discontinued after stage I, and the specimen well irrigated with Ringer solution, the diffraction pattern changed to one which resembled fresh nerve pattern, but showed an appreciable general increase in spacing (e.g. from 85.9 ± 1.5 Å to about 98 Å, with corresponding increases in other spacings). This treatment with Ringer solution did not restore the activity of the nerve. When the alcohol treatment was continued to the end of stage II, and the specimen then well-irrigated with Ringer solution, the diffraction pattern obtained showed two well-defined bands of moderate intensities at about 110 Å and 55 Å. The first of these bands was the stronger. In addition, some patterns showed faint bands at about 70 Å and 40 Å.

In six experiments, treatment with alcohol was continued into stage II and dry air was then blown into the cell in order to drive off the solvent. After a few hours the diffraction patterns were examined. All patterns showed a sharp and intense band at 47.2 ± 1.0 Å, and a very faint band at 90 to 100 Å. Three of the patterns showed a band at 57.6 ± 1.1 Å which was of a similar intensity to the 47.2 ± 1.0 Å band, whilst in the other three a less intense band at 61.5 ± 0.5 Å replaced the band at 57.6 ± 1.1. The more intense patterns also showed weak bands at 35.7 ± 1.2 Å, and at about 17 Å.

If the alcohol-treated specimen were treated with Ringer solution before drying, the relative intensity of the band at 90 to 100 Å was markedly increased.

When the above experiments were repeated using acetone instead of alcohol, changes in diffraction pattern were observed which were very similar to those seen in stage I of the alcohol treatment (Fig. 3). At this stage, the patterns showed an intense band at 52.2 ± 0.7 Å, a less intense band at 43.2 ± 1.8 Å, and a faint band at about 90 Å (Fig. 3b). On further

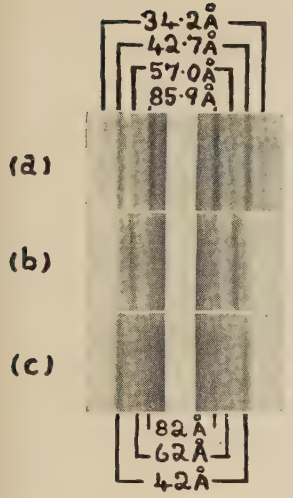

Fig. 3.

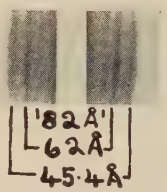

Fig. 4.

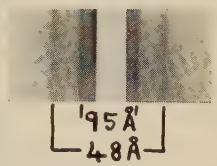

Fig. 5.

Fig. 3. Examples of the diffraction patterns obtained during the treatment of fresh nerve acetone vapour. (a) Fresh nerve pattern. (b) and (c) Patterns obtained during treatment with acetone vapour.
Fig. 4. X-ray diffraction pattern obtained after treatment of fresh nerve with acetone vapour and subsequent drying.
Fig. 5. X-ray diffraction pattern obtained after treatment of fresh nerve with acetone vapour and subsequent treatment with Ringer solution.

treatment with the acetone vapour, the diffraction pattern changed to one showing a relatively strong band at about 42 Å, and weak bands at about 62 Å and 82 Å (Fig. 3c). On drying, the pattern underwent further slight changes, the lower spacing increasing to 45.4 ± 1.0 Å, and the relative intensities of the diffraction bands undergoing some readjustments (Fig. 4). When the acetone treated specimen was re-wetted with Ringer solution, the pattern changed to one showing two well-defined bands at about 48 Å and 95 Å (Fig. 5).

Specimens treated with Ringer solution before drying gave a pattern very similar in appearance to that obtained from specimens which had been dried immediately after the acetone vapour treatment.

A slow stream of air passing through ether and into the cell had no immediate effect on the diffraction pattern of fresh, Ringer-irrigated, nerve or on the action potentials. A rapid stream of ether vapour appeared to dry the specimen, the changes in diffraction pattern following a similar sequence to that of normal drying (2).

2) *Ringer solution containing lipid solvents.* Twelve experiments, involv-

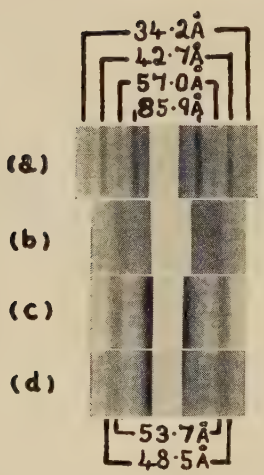

Fig. 6.

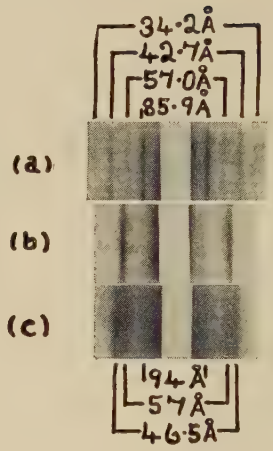

Fig. 7.

Fig. 6. Examples of diffraction patterns obtained during treatment of fresh nerve with varying proportions of alcohol and Ringer solution. (a) Fresh nerve pattern. (b) Fresh nerve after immersion for twelve hours in a 1:1 alcohol-in-Ringer solution. (c) Fresh nerve after immersion for twenty-four hours in a 2:1 alcohol-in-Ringer solution. (d) Fresh nerve after about twenty-four hours in a 3:1 alcohol-in-Ringer solution.

Fig. 7. Examples of diffraction patterns obtained during treatment of fresh nerve with varying proportions of acetone and Ringer solution. (a) Fresh nerve pattern. (b) Fresh nerve after immersion for four hours in a 3:1 acetone-in-Ringer solution. (c) Fresh nerve after 4 hours' exposure to pure acetone.

ing a total of fifty exposures, were carried out with varying concentrations of ethyl alcohol in Ringer solution. Six series of experiments were carried out using acetone in Ringer.

When fresh nerve was immersed in a medium consisting of one part of ethyl alcohol and three parts Ringer solution, the intensity of the 57.0 ± 1.5 Å band of the fresh nerve pattern increased relative to the other bands, until, after about twenty hours, it was of the same order as that of the next lower spacing (corresponding to the 42.7 ± 1.0 Å spacing in the fresh nerve pattern). This change was accompanied by a slight general increase in spacings.

In a mixture containing a 1:2 proportion of alcohol and Ringer solution, this general increase in spacings became more marked, and the intensity of the band seen originally at 57.0 ± 1.0 Å in fresh nerve now became comparable with that of the higher spacing (85.9 ± 1.5 Å in the fresh nerve pattern).

In a 1:1 alcohol–Ringer mixture the fresh nerve pattern was eventually

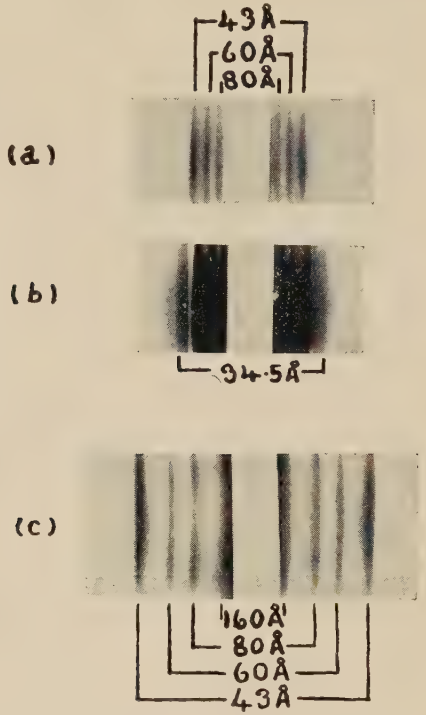

Fig. 8. X-ray diffraction patterns obtained after immersion of fresh nerve for twenty-four hours in a 1:3 acetone-in-Ringer solution, and subsequent drying to constant weight. In (a) and (b) the centre of the film has been masked with a thin sheet of aluminium foil to reduce halation from higher spacings.

replaced by a pattern which showed an intense band at about 54 Å, and very faint bands at about 110 Å and 43 Å (Fig. 6b).

In solutions containing greater proportions of alcohol, the faint bands were no longer observed, and the intense band was replaced by two well-defined and equally intense diffractions at 48.5 ± 0.5 Å and 53.7 ± 0.6 Å (Fig. 6c and d). In absolute alcohol the diffraction pattern showed only a very faint band at about 42 Å.

When similar experiments were carried out with acetone in Ringer solution, the initial changes in diffraction pattern were very much like those observed with alcohol–Ringer mixtures. After four or five hours in a 3:1 mixture of acetone and Ringer solution, the diffraction pattern showed an intense band at about 53 Å and a faint one at about 110 Å (Fig. 7b). In pure acetone the pattern showed an intense band at about 46.5 Å, and progressively weaker ones at about 57 Å and 94 Å (Fig. 7c).

Specimens immersed for twenty-four hours in a mixture containing 1:2 or 1:3 proportions of alcohol (or acetone) and Ringer solution and subse-

quently dried, gave a diffraction pattern which showed bands at approximately 34.5 Å, 43.0 Å, 60.0 Å, 80.0 Å and 160 Å (Fig. 8). A similar pattern could be obtained by treatment with solutions containing greater proportions of solvent for short periods of time. In this pattern the three lower spacings coincided with those found in normal dried nerve, but in the solvent-treated specimen the 60 A band appeared relatively much more intense than in the normal. Also, the relative intensities of the 80 and 160 A bands appeared to differ appreciably from those of the corresponding 73.5 ± 1.5 Å and 143.5 ± 3.0 Å bands in the normal dried nerve pattern. When the treatment with low proportions of solvent in Ringer solution was carried out at low temperatures (0 to 3° C) the dried specimen gave a pattern which was identical with that of normal dried nerve.

The diffraction patterns obtained from nerve specimens which had been treated with Ringer solution containing the higher proportions of solvent for periods up to twenty-four hours and dried subsequently, showed further increases in the higher spacings (i.e above 70 Å) and corresponding decreases in intensities. With alcohol, these bands eventually became too weak to be detected. In some of these patterns spacings of the orders of 46 to 48 Å and 53 to 55 Å were often found to replace the more usual ones of 43.5 ± 1.0 Å and 56.5 ± 1.0 Å or 60.0 ± 1.0 Å. These spacings could be reduced to the normal values (as in dried nerve) by re-immersing the specimens in Ringer solution and then re-drying. This treatment did not reduce the higher spacings (i.e. those above 70 Å), but it was often observed to increase their intensities.

3) *Pure solvents.* When alcohol was refluxed over normal dried nerve, the bands of the dried nerve pattern became progressively more faint and diffuse; eventually there remained only a faint general scatter from 40 Å to 60 Å. When refluxing was discontinued, the first definite diffraction band to reappear was at about 37 to 38 Å. This was initially rather diffuse, but the definition gradually improved as the intensity increased. At the same time the spacing increased to about 44 Å (Fig. 9). Before these changes were completed, a further diffraction band had appeared at about 58 Å, and this too became sharper, the spacing increasing steadily to about 62 Å. When the specimen was dried to constant weight, the long spacing diffraction pattern (Fig. 10a) showed intense diffractions at 43.6 ± 1.2 Å and 62.6 ± 1.4 Å, and well defined but faint ones at 34.8 ± 1.0 Å and at about 17.2 Å. A few patterns showed a band at about 48 A instead of at 43.6 ± 1.2 Å, but this could be reduced to the more usual value by immersing the specimen in Ringer solution for about thirty minutes and then re-drying. As compared with

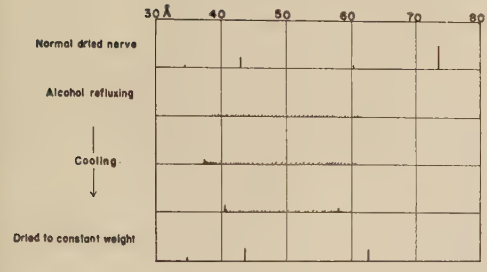

Fig. 9.

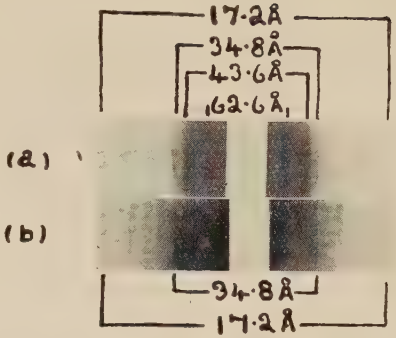

Fig. 10.

Fig. 9. Summary of changes in diffraction patterns observed during cooling following a 20 minute period of refluxing with alcohol.
Fig. 10. Effect of alcohol on the X-ray diffraction pattern of dried nerve. (a) Pattern obtained after refluxing with alcohol for 5 minutes, and subsequent drying. (b) Slow refluxing repeated after treatment as in (a). Pattern obtained during the first 15 minutes of refluxing.

the pattern of normal dried nerve, there appeared to be appreciable increases in the intensities of the bands at 62 Å, 35 Å, and 17 Å, relative to the one at 43 Å. Spacings above 70 Å were no longer observed. When alcohol was once more refluxed over the specimen, the diffraction bands again became faint and diffuse, the 43.6 ± 1.2 Å and 62.6 ± 1.4 Å bands being affected before the ones at 34.8 ± 1.0 Å and 17.2 (Fig. 10b). Continued refluxing resulted in a gradual decrease in intensity of all the diffraction bands in the pattern of the dried specimen. Again it was observed that in cases where a faint diffraction band persisted in the region of 90 to 100 Å, its intensity could be increased by immersing the specimen in Ringer solution, and then re-drying. After prolonged refluxing with alcohol (i.e. when the higher spacings could no longer be detected in the diffraction of the dried specimen), this treatment had no effect.

Refluxing acetone over dried nerve resulted in the complete disappearance of the 60 Å band (Fig. 11b). The 34.5 ± 0.5 Å band appeared to be relatively unaffected, the 43.5 ± 1.0 Å and 73.5 ± 1.5 Å spacings being reduced by about 3 A during refluxing, but returning to their normal values when refluxing was discontinued. Refluxing was maintained for two hours without appreciably affecting the definition of these spacings. The 60.0 ± 1.0 Å band reappeared on cooling.

When dried nerve was suspended in the distillation cell over ether, the intensity of the 60.0 ± 1.0 band was observed to decrease even before distillation commenced. On refluxing, the 60.0 ± 1.0 Å band disappeared completely,

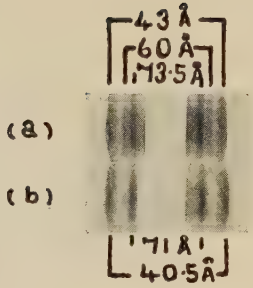

Fig. 11.

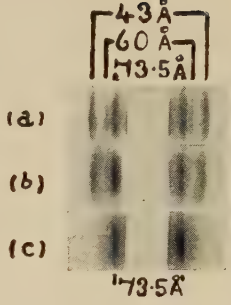

Fig. 12.

Fig. 11. Effect of acetone on the X-ray diffraction pattern of dried nerve. (a) Normal dried nerve pattern. (b) Pattern obtained during the refluxing of acetone over the specimen. The exposure (of 15 min. duration) was started after 5 min. of preliminary refluxing.

Fig. 12. Effect of ether on the X-ray diffraction pattern of dried nerve. (a) Normal dried nerve. (b) Pattern obtained after suspending dried nerve over ether in the distillation cell for 12 hours. (c) Pattern obtained during refluxing. Exposure (of 15 min. duration) started after 5 min. of preliminary refluxing.

and after about thirty minutes, the 43.5 ± 1.0 Å band became faint and diffuse (Fig. 12c). The 73.5 ± 1.5 Å band was unaffected by ether in these experiments. When refluxing was discontinued, the definition of the 43.5 ± 1.0 Å band improved again, and the intensity increased. The 60.0 ± 1.0 Å band did not reappear until the specimen had been taken out of the cell, and left in air for an hour. Continued refluxing resulted in the complete disappearance of the 34.5 ± 0.5 Å band from the pattern of dried specimen, and a marked increase in the intensity of the band at 43.5 ± 1.0 Å. The treatment with ether was repeated under carefully controlled conditions in a separate distillation apparatus. The temperature of the solvent in contact with the specimen was kept at 12° C. From time to time the distillation apparatus was opened

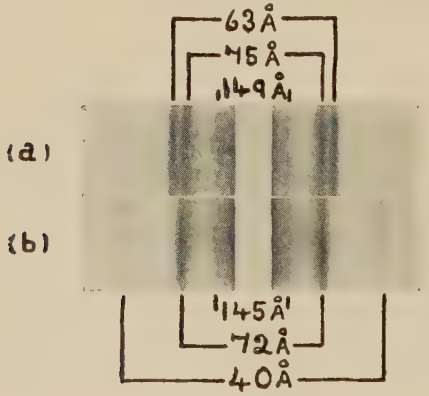

Fig. 13. (a) X-ray diffraction pattern obtained after refluxing dried nerve with ether for 24 hours, and subsequent drying. (b) Specimen as in (a), when heated to 100° C.

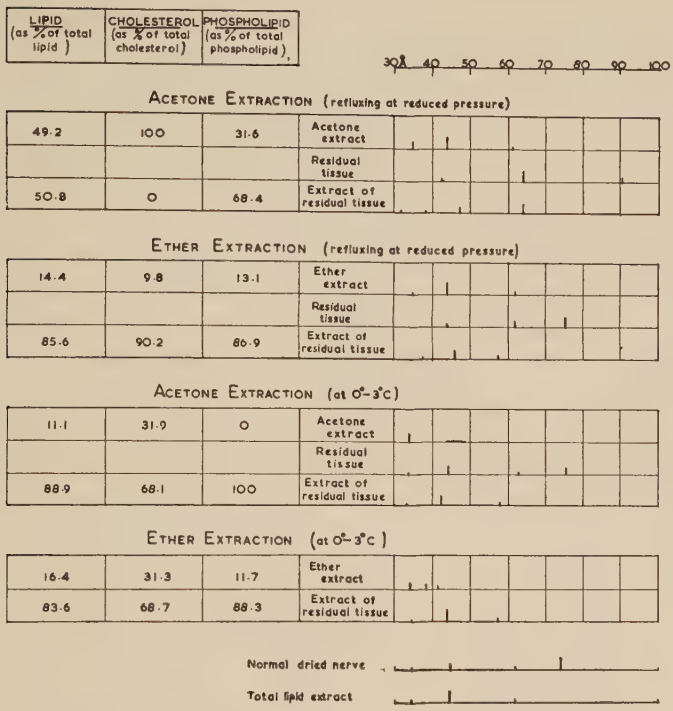
LIPID (as % of total lipid)
CHOLESTEROL (as % of total cholesterol)
PHOSPHOLIPID (as % of total phospholipid)
30Å 40 50 60 70 80 90 100
ACETONE EXTRACTION (refluxing at reduced pressure)
49·2 100 31·6 Acetone extract
Residual tissue
50·8 0 68·4 Extract of residual tissue
ETHER EXTRACTION (refluxing at reduced pressure)
14·4 9·8 13·1 Ether extract
Residual tissue
85·6 90·2 86·9 Extract of residual tissue
ACETONE EXTRACTION (at 0°–3°C)
11·1 31·9 0 Acetone extract
Residual tissue
88·9 68·1 100 Extract of residual tissue
ETHER EXTRACTION (at 0°–3°C)
16·4 31·3 11·7 Ether extract
83·6 68·7 88·3 Extract of residual tissue
Normal dried nerve
Total lipid extract

Fig. 14. Summary of the effects of partial lipid extraction on the X-ray diffraction pattern of nerve.

up, and a segment of nerve removed. This was dried, and the X-ray diffraction pattern recorded. After about twenty-four hours' refluxing, the pattern consisted of two sharp and intense bands at about 75.5 Å and 60 Å, and a fainter band at about 148 Å (Fig. 13a). Continued treatment under these conditions did not appear to effect any further changes. The effect of heating on the pattern obtained after treating with ether for twenty-four hours is shown in Fig. 13. The band at about 63 Å was replaced by a less intense diffraction at about 40 Å, but the higher spacings remained unaffected. This effect was completely reversible.

A number of experiments were carried out in which nerve was partially extracted, and the diffraction patterns of both the residual dry tissue and the dried lipid extract were examined. The extraction was subsequently completed, and the diffraction pattern of this second lipid extract was also studied. The amount of lipid extracted, and the proportions of cholesterol and phospholipid in each specimen were determined. A summary of the findings is given in Fig. 14. At room temperature and reduced pressure, acetone and

ether appeared to extract lipids in proportions corresponding approximately to the relative intensities of the diffraction bands in the dried nerve pattern. At low temperature (i.e. below 3° C), acetone and ether extracted cholesterol preferentially. Alcohol would extract practically all the lipid, but when diffraction patterns (of a dried specimen) were recorded after only part of the lipid had been removed, the 60.0 ±1.0 Å band was much more intense than in the pattern of normal dried nerve.

In the experiments in which fresh nerve was treated with solvent vapours and dried nerve with refluxing solvents, interference from the diffraction of the cellophane (or polythene) windows of the observation cells made an interpretation of the short spacing patterns impracticable.

When small amounts of solvent were added to the Ringer solution in which fresh nerve was immersed, the short spacing ring (4.7 Å) showed no appreciable change. When high proportions of solvent (more than 50 per cent) were used, a sharp diffraction ring at 4.2 Å was superimposed on the diffuse 4.7 Å ring. When such specimens were subsequently dried, the sharp 4.2 Å ring became much more marked.

DISCUSSION

The diffraction patterns of fresh nerve, normal dried nerve, and total lipid extract of nerve, and the effect of drying and temperature on such patterns, have been discussed elsewhere (1, 2, 3, 4). These findings indicated the existence in myelin of a highly ordered lipid-lipoprotein unit. Present results are in keeping with this hypothesis.

Small amounts of alcohol and acetone vapours have an almost immediate effect on action potentials and the diffraction patterns of irrigated fresh nerve, whilst ether has no such immediate effect. This is presumably due to the complete miscibility of water with acetone and alcohol but not with ether.

The initial effect of alcohol is probably one of dehydration. This effect is reflected in the initial shrinkage of all spacings, and in the increase in intensity of the diffraction band corresponding to the 57 ±1.5 Å band in the fresh nerve pattern, a change very similar to those seen in the initial stages of normal drying. These changes may be due to either an extensive rearrangement of the diffracting groups in the original unit, or to the break-up of the unit. The apparently independent variation both in position and intensity of some of the diffraction bands during exposure to alcohol, suggests the separation of two or more independent components during some stages of the treatment. Previous data (2, 4), suggested the existence in dried nerve of

separate lipid phases and a residual lipo-protein. A similar separation of lipid phases may be brought about by treatment with solvents. However, the effect of alcohol appears to go further in its modification of structure than does normal drying. Even at the end of stage I, the alcohol-treated specimen shows a diffraction band at 91.5 ±4.0 Å, which when the specimen is dried, gives rise to a faint band at 90 to 100 Å (as compared with the much more intense band at 73.5 ±1.5 Å found in normal dried nerve). The total effect of stage I of treatment of fresh nerve with alcohol vapour appears to be a dehydration, accompanied by a greater modification of the residual lipo-protein complex (as indicated by the higher spacings) than is brought about by normal drying. Further modifications of this complex probably occurs in stage II. The diffraction patterns during this stage show some resemblance to those obtained from dried specimens which have been refluxed with alcohol, and examined whilst some of the alcohol remained in the structure. Thus it is quite possible that stage II of the alcohol vapour treatment reflects a penetration, and partial solvation, of some of the lipid components.

When alcohol was added to the Ringer solution and allowed to act on fresh nerve, the sequence of changes observed resembled that seen when alcohol vapour was used. In absolute alcohol, however, the nerve specimen showed only one diffraction band at about 42 Å. This probably indicates a more extensive penetration, or a more complete solvation than is possible using alcohol vapour. Moreover the low intensity of the bands above 70 Å appears to indicate a still further modification of the lipo-protein complex.

The increase in intensity of the 90 to 100 Å band in specimens previously exposed to absolute alcohol and then treated with Ringer solution and dried subsequently, probably reflects a recrystallisation of the residual lipo-protein complex. When such treatment has resulted in the complete disappearance of the higher spacings, treatment with Ringer solution has no appreciable effect.

The gradual decrease in intensity of the bands below 70 Å after refluxing and drying, probably reflects the extraction of lipid components.

The changes brought about by acetone are similar to those observed during the first stages of alcohol treatment. However, its effect on the spacings above 90 Å is not so marked. After stage I, an intense band appears at about 42 Å, just as in the later stages of normal drying. It would therefore appear that acetone vapour simply brings about a dehydration without any indication of penetration into the free lipid components (as represented by the 42 and 62 Å bands). However, when nerve was treated with liquid acetone, the diffraction pattern showed a band at about 90 Å which decreased

to about 82 Å when the acetone was evaporated. Acetone thus apparently will penetrate the lipo-protein complex, at least when it is applied to fresh nerve, and modify the spacing of the complex which in normal dried nerve has the value of 73.5 ± 1.5 Å.

The changes brought about by refluxing acetone over dried nerve could probably be explained in terms of temperature effects alone. Previous experiments on the effects of temperature on the diffraction pattern of dried nerve have shown that all spacings show a contraction with rise in temperature above 20° C, and that the intensity of the 60.0 ± 1.0 Å band falls rapidly when the specimen is heated.

Refluxing ether over dried nerve does not involve any appreciable increase in temperature. Thus the effects observed on the 43.0 ± 1.0 and 60.0 ± 1.0 Å bands must be due to penetration of the ether into these particular components. In the limited time in which the ether was refluxed over the dried nerve, there was no change in the complex spacing (73.4 ± 1.5 Å). Even on prolonged treatment with ether (100 hrs.), the bands at 73.5 ± 1.5 Å and 143.5 ± 3.0 Å in the dried nerve pattern were relatively unaffected. However, the 60.0 ± 1.0 Å band became much more intense than in the normal dried nerve pattern and the 43.5 ± 1.0 Å band disappeared entirely. This change is similar to the one observed when dried nerve was cooled to about —20° C, and which may be due to the existence of two polymorphic forms of a lipid component, the one showing a predominant band at about 40 Å, and the other at about 60 Å. The effect of ether might thus be interpreted as a transition from the 40 Å form to the 60 Å form. The 63 Å band was replaced by one at about 40 Å on heating the ether-treated specimen to about 100° C, but on cooling the process was completely reversed. If the initial effect of ether had been merely to recrystallise the lipid component, one would have expected the effect to disappear after subsequent heating and cooling, and the resulting pattern to resemble that of normal dried nerve. The fact that this is not the case suggests that ether may actually change the composition of the lipid component.

The bands occasionally observed at 53 to 55 Å and 46 to 48 Å in the patterns of specimens which had been treated with solvents before drying, probably indicate further polymorphic forms of the main 40 to 60 Å lipid component.

Possible polymorphic transitions may also in part, explain some of the changes observed during partial extraction of nerve by lipid solvents. In many of the patterns of residual tissue, the relative intensity of the 60.0 ± 1.0 Å band is unusually high, and though preferential extraction may be part of

the explanation (particulary in the case of the early disappearance of the diffraction band at 34.5 ±1.0 A), the principal effect on the 60.0 ±1.0 Å and 43.5 ±1.0 Å spacings is a shift in emphasis between them. Another possibility is the partial breakdown of the lipo-protein complex, with consequent liberation of lipid components, and affecting the relative intensities of the bands below 70 Å (the "free lipid" bands). Thus, in partial extraction of nerve, three factors may be involved: the preferential extraction of lipid, a polymorphic transition from one lipid form to another, and a liberation of fresh lipid from a lipoprotein complex.

SUMMARY

1. The effects of ethyl alcohol, ether, and acetone on the X-ray diffraction patterns of fresh nerve and dried nerve have been studied.

2. Ethyl alcohol and acetone vapours produced rapid changes in the diffraction pattern of fresh nerve. Ether vapour had relatively little effect.

The effects of mixtures containing varying proportions of alcohol or acetone in Ringer solution were similar to those of the solvent vapours.

Ethyl alcohol, acetone, and ether, all readily affected the spacings below 70 A in the dried nerve pattern. The spacings above 70 Å were much more susceptible to alcohol treatment than to ether and acetone.

3. The results are in keeping with the existence in myelin of a highly-ordered lipid-lipoprotein unit.

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EFFECTS OF PROTEOLYTIC ENZYMES ON THE FERTILIZATION MEMBRANE AND JELLY LAYERS OF THE AMPHIBIAN EMBRYO

P. L. TOWNES[1]

Department of Biology, University of Rochester, Rochester, N.Y.

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Although a great deal of information concerning the fertilization membrane and extraneous layers of the echinoderm egg is available (11), the amphibian egg has been somewhat neglected in this regard. Chemical analysis of the jelly of the amphibian egg (13), has indicated that this structure is at least partly mucoprotein in composition. Cooper (3) clearly demonstrated the presence of a "hatching secretion" in *Rana pipiens*, but did not attempt to characterize it more specifically than its ability to remove the extraneous layers. A very brief report by Bliss (2) showed that removal of these layers may be achieved through the application of trypsin. The investigations to be reported here provide some information on the chemical composition of these structures as revealed by their reaction to various enzymes.

MATERIAL AND METHODS

Early developmental stages of *Triturus torosus*, *Amblystoma punctatum*, *Pleurodeles waltlii*, and *Rana pipiens* were exposed to varying concentrations of trypsin, chymotrypsin, ficin, papain, hyaluronidase, mycozyme (protease) and erepsin in Holtfreter's Standard Solution[2]. The activity of these enzymes as regards their effectiveness in removing the fertilization membrane and outer jelly was recorded. Subsequent development of the embryos was observed to detect possible deleterious effects of the treatment. In most cases the animals were cultured in syracuse dishes. The jelly was removed mechanically, only when the effects on the membrane were considered exclusively.

RESULTS

Table I summarizes the effects of crystalline trypsin and chymotrypsin on the fertilization membrane of *Triturus torosus* at Harrison stage 13 (early

[1] Public Health Service Research Fellow of the National Cancer Institute.

[2] We are much indebted to Dr. A. W. Allen of the Schering Corp. who kindly supplied samples of Hyronase (Hyaluronidase). The crystalline salt-free trypsin and chymotrypsin were obtained from Worthington Biochem. Labs. The commercial preparations of ficin, erepsin and mycozyme were obtained from General Biochem. Labs., and papain from Nutritional Biochem. Corp.

medullary plate). It will be noted that the time required for the removal of the membrane is roughly inversely proportional to the concentration. In all cases the initial response of the membrane to the enzyme is a pronounced swelling, followed by a rupture and slipping off which may last for a minute, and finally complete solution of the membrane within a few minutes thereafter. In this series the denuded embryos were left exposed to the enzymes for four days, by which time they had reached stage 28. As compared to normal controls, neither rate of development nor morphogenesis of these embryos were noticeably affected. Experiments performed on wound healing showed that this process was not seriously retarded, and single cells cultured in these solutions for 6 to 7 days exhibited no injurious effects as judged by cellular divisions and motility.

Some information regarding the effectiveness of these enzymes on different developmental stages was also obtained. Table II shows in a summary fashion that there are no marked differences in this respect. The stages used in this experiment were early gastrula, early neural plate and tail bud, stages 10, 13 and 19 respectively. It may be noted that if one compares the relative effectiveness of the two enzymes in Tables I and II, one finds in all instances that chymotrypsin is more effective than trypsin. Continued exposure to the concentration reported in Table II was found to selectively digest the "surface coat" promoting cellular disaggregation; however this did not become manifest until approximately twenty hours after membrane removal.

Further experiments using several stages ranging from the fertilized egg to the pre-hatching stage of *Pleurodeles waltlii* gave further support to the notion that there is little or no difference in susceptibility of the membranes at different stages of development. This species was found to be more resistant in general, since it required 5 to 6 hours to remove the membrane with 400 mg per cent chymotrypsin, but this requirement was found to be essentially unchanged in all stages used. In this series it was found that the jelly was removed in 1 1/2 hours at the same concentration, and development was normal for about two days. Similar results were obtained with the unfertilized egg, fertilized egg, gastrula and neurula stages of *Rana pipiens*. Attempts to fertilize the enzymatically denuded eggs were unsuccessful. It was found that 400 mg per cent chymotrypsin would completely free embryos from all external coverings after six hours.

The results obtained with non-crystalline enzymes were briefly as follows:

Ficin at pH 6.5 was applied to *Amblystoma* neurulae at concentrations from 1 per cent through a graded series of dilutions down to 0.13 per cent. Proportional to the enzyme concentrations the fertilization membrane dis-

TABLE I

Effects of different concentrations of trypsin and chymotrypsin on the removal of the fertilization membrane in *Triturus torosus* (stage 13).

Trypsin pH 7.8 in mg per cent	Time for complete removal of membrane in hours	Chymotrypsin pH 8.0 in mg per cent	Time for complete removal of membrane in hours
300	1 1/2	200	1/2
200	1 1/2	166	1/2
150	2 1/2	100	1
100	3	66	1
12.5	20	33	2

TABLE II

Effects of trypsin and chymotrypsin on different stages of *Amblystoma punctatum.*

Trypsin pH 6.8 uniform conc. 800 mg per cent	Time for complete removal of membrane	Chymotrypsin pH 6.8 uniform conc. 800 mg per cent	Time for complete removal of membrane
Stage 10	3 hours	Stage 10–11	10 minutes
Stage 13	3 hours	Stage 13	12 minutes
Trypsin and Chymotryp.		Stage 19	10 minutes
Stage 13	15 minutes		

appeared within periods ranging from 25 minutes to 4 1/2 hours. Development in lower concentrations was normal for two days, although exposure to higher concentrations resulted in a softening of the "coat".

Mycozyme at pH 7.1 was able to remove the membrane from *Amblystoma* neurulae in 6 hours at 1 per cent, in 7 hours at 0.80 per cent and in 9 hours at 0.66 per cent. Development was apparently normal after 24 hours exposure to the enzyme. Neither the jelly nor the capsule (the firm transparent layer immediately inside the jelly) were digested by this enzyme.

Papain at pH 6.5, activated with small quantities of cysteine hydrochloride, was similarly able to remove the membrane from *Amblystoma* neurulae in 30 minutes at 1 per cent, and in 60 minutes at 0.5 per cent. Still lower concentrations were ineffective even after 6 hours. In addition, it was found that at a concentration of 1 per cent, the jelly was dissolved in 45 minutes with the exception of the inner capsule which remained resistent.

In comparable experiments on *Triturus torosus* neurulae the membrane disappeared in 15 minutes in a solution of 1 per cent papain, and in 35

minutes in a 0.5 per cent solution. It was possible to obtain completely freed embryos from intact egg clusters of *Triturus* neurulae exposed to concentrations of 1–3 per cent papain for 6–10 hours, which upon removal from the enzyme solution developed normally. In this case the enzyme was able to digest all of the membranes and jelly external to the embryo. Observations on the process of dissolution of the jelly of *Triturus* revealed the presence of fibrous thread-like processes radially arranged throughout the jelly and being anchored to the inner capsule. These threads are not visible in the intact jelly and appear for a limited time during the initial breakdown, after which they are likewise destroyed.

Hyaluronidase in activities of 600 TRU and lower was totally ineffective on all structures. This is somewhat surprising considering that the available data on the composition of the jelly (13) suggest that it is a mucoprotein. If one considers, however, the diverse types of mucoproteins and the different specificities of hyaluronidase preparations (7, 12) our negative findings in this connection cannot be interpreted as disproving the existence of a mucoprotein component.

Erepsin had no effect at concentrations of 1 per cent and lower for periods up to 48 hours.

DISCUSSION

The above results indicate that the fertilization membrane of these amphibian species is subject to enzymatic proteolysis in early developmental stages, i.e. at a period long before the membrane is normally softened by some secretions of the animal proper, which are possibly derived from the "frontal glands" described by Holtfreter (4). This suggests that the fertilization membrane is predominantly composed of protein. Considering the high specificity of trypsin and chymotrypsin (1), one may infer that peptide linkages involving arginine or lysine and tyrosine or phenylalanine are present. The greater efficiency of chymotrypsin in the proteolytic degradation suggests that tyrosine or phenylalanine residues occupy a position of significance for the integrity of this structure.

The susceptibility of the membrane to chymotrypsin and trypsin is similar to the results obtained on the echinoderm except that in the amphibian egg disulphide linkages do not appear to be as significant. In this respect, the fertilization membrane seems to be similar to the vitelline membrane of the echinoderm prior to its hardening or keratinization (8, 12). Although the various amphibian species differed considerably with respect to the susceptibility of

the fertilization membrane and jelly to the enzymes, in all of them the capsule was the least susceptible structure. Particularly in the case of *Amblystoma punctatum* the capsule was extremely resistant. Nevertheless, paper partition chromatographic analysis performed in collaboration with T. Koszalka in this laboratory has shown that the capsule does contain a wide array of amino acids. This suggests that the resistance of the capsule to enzymatic removal may be due to the fact that the protein configuration of this structure differs from that of the more susceptible structures.

The rupture and slipping off of the fertilization membrane is in agreement with the observations on the hatching echinoderm (5, 6, 12). However, whether this rupture occurs at any predetermined weak point or natural site of breaking is difficult to ascertain.

It is interesting to note that relatively high concentrations of crystalline trypsin and chymotrypsin (Table I) fail to produce any harmful effects either on the whole embryos or their isolated cells. At the higher concentration reported in Table II, the amphibian coat is selectively digested resulting in cellular disaggregation. This action on the coat will be considered more extensively in another context. The free cells do not appear to be subject to enzymatic proteolysis and may be cultured in the enzyme solutions for 6 to 7 days, during which they exhibit normal motility. The resistance of living cells to digestion is in agreement with the concepts of Northrop (10). This does not exclude the possibility that enzymes may actually enter and affect living cells, as recently suggested by Moore (9). In support of this idea we have observed that 400 mg per cent chymotrypsin produces a decrease in intensity of surface pigmentation in early cleavage stages of *Rana pipiens,* but not in other species.

Contrary to embryonic cells which have been disaggregated by alkali, the enzyme-dispersed cells resist strongly a re-aggregation in an otherwise favorable medium, indicating that the coat as well as the factors involved in cellular adhesion are at least partly of a protein nature. The non-crystalline enzymes, possibly because of impurities, were found to be more injurious than the crystalline enzymes. Embryos continuously exposed to the former rarely survived more than 2 days.

SUMMARY

The effects of several enzymes on the fertilization membrane and jelly layers of the amphibian embryo have been studied. Their removal by proteolytic enzymes provides evidence of their protein nature and furnishes

some information concerning the types of peptide bonds which are essential for their integrity.

For experimental procedure in general, proteolytic enzymes may provide a convenient method for the removal of these structures where denuding of the eggs by mechanical means is difficult.

I wish to express my gratitude for the continued encouragement and many helpful suggestions of Dr. J. Holtfreter. I am also grateful to Mr. T. Koszalka for his kind aid in the chromatographic analysis.

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A LIPOPROTEIN OF RAT LIVER NUCLEI[1,2]

T. Y. WANG, D. T. MAYER and L. E. THOMAS

Departments of Biochemistry and Agricultural Chemistry, University of Missouri, Columbia, Miss.

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Cellular proteins insoluble in water or salt solutions but soluble in alkaline solutions have been reported several times. Bensley and Hoerr (5) obtained preparations from rabbit and guinea pig liver which they named "ellipsin". The material was precipitated by the addition of acetic acid to the alkaline solution and was reported to contain 30 per cent "fatty substances". Luck (10) obtained an alkali-soluble protein fraction from dog liver. This material, designated "globulin II", was precipitated by the addition of acid. These preparations probably contained proteins from the cytoplasm and nucleus. (We have found such proteins in both cytoplasmic and nuclear fractions of rat liver.)

Mayer (11) reported the presence of an alkali-soluble protein fraction in the nuclei of calf thymus, isolated by a modified Behrens technique. It was precipitated at pH 5.8 to 6.2 by acetic acid. Boshijan (6), Mayer and Gulick (12), Stedman and Stedman (16, 17, 18), Jeener (8), and Zbarski and Debov (23) have described similar preparations. Two of the present authors (21) described a protein fraction extracted from boar spermatozoa with alkali and precipitated at about pH 6 with acetic acid. A preliminary report (22) was also made indicating that this material could not be obtained from the tail-midpiece fraction of boar spermatozoa, but only from the heads and that similar proteins had been obtained from rat liver nuclei and "isolated chromosomes" of calf thymus. In the latter case it was found (9) that the "residual chromosomes", prepared by the method of Mirsky and Ris, consist almost entirely of this protein fraction.

Evidence has now been obtained indicating that the proteins insoluble in neutral salt solutions, but soluble in alkaline solutions, obtained from the nuclei of rat liver, calf liver, calf thymus, ox spleen and chicken erythrocytes and from the heads of bull sperm all have tightly bound lipid, removable

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by hot alcohol but not by ether. The present paper reports some properties of the alkali-soluble lipoprotein of rat liver nuclei.

EXPERIMENTAL METHODS AND RESULTS

Rat liver nuclei were isolated by the method of Dounce (7), modified slightly, or by Behrens' method (2), also modified somewhat.[1] In either procedure, the liver was frozen as soon as possible after the animals were killed and all operations used in isolating nuclei and extracting proteins (except the lyophilizing in the Behrens method) were carried out at 0°—4° C.

The nuclei were extracted exhaustively with 1 M sodium chloride solution to remove the nucleohistone, using, in each extraction, approximately 100 ml of solution for each 5 ml of packed nuclei. Ordinarily, three extractions were sufficient for the nuclei prepared by the procedure of Dounce. The nuclei obtained by the Behrens method required more extraction due to the formation of a more viscous solution at the outset. The first centrifugation was carried out at 4 500 r.p.m. for about three hours with shorter periods for subsequent centrifugations.

After exhaustive extraction with sodium chloride some of the nuclei retained their original shape and size and the nuclear membrane of those nuclei appeared to be intact. The nuclear contents appeared to have been mostly removed, and the "ghost" nuclei were clumped. Many of the nuclei were broken into fragments.

The nuclei were then stirred three times with 0.1 N sodium hydroxide solution (about 100 ml of solution for each 5 ml of packed nuclei) for 30 minute periods, the mixture being centrifuged each time at 4 500 r.p.m. until a clear solution was obtained. From 3 to 5 hours were required. The pH of the extract was approximately 12.5. Extraction can be carried out at a lower pH, but the lipoprotein goes into solution much more slowly under those conditions, its solubility apparently being quite low over a wide pH range. It was therefore deemed better to extract for a shorter period at the higher pH, as described.

Acetic acid was added to the extract slowly and a precipitate, having the characteristics of a lipoprotein, was obtained at pH 5.7–6.0. It was collected by centrifugation at about 3 000 r.p.m. for 10 minutes. The precipitate was dissolved in 0.1 N sodium hydroxide and reprecipitated with acetic acid three times. The alkaline solution was centrifuged each time to remove any insoluble material before the reprecipitation. The material prepared in this way is referred to below as purified lipoprotein.

Ultraviolet light absorption by an alkaline solution of the precipitated lipoprotein fraction showed no indication of the presence of nucleic acid,

[1] In the Behrens procedure we used a mixture of benzene and carbon tetrachloride of low specific gravity to suspend the lyophilized tissue for grinding, and mixtures of these two liquids of increasing specific gravity for the differential centrifugation. A detailed description is not given here because the proportions of the two liquids used must necessarily be varied somewhat each time the procedure is used to obtain a good separation of the nuclei.

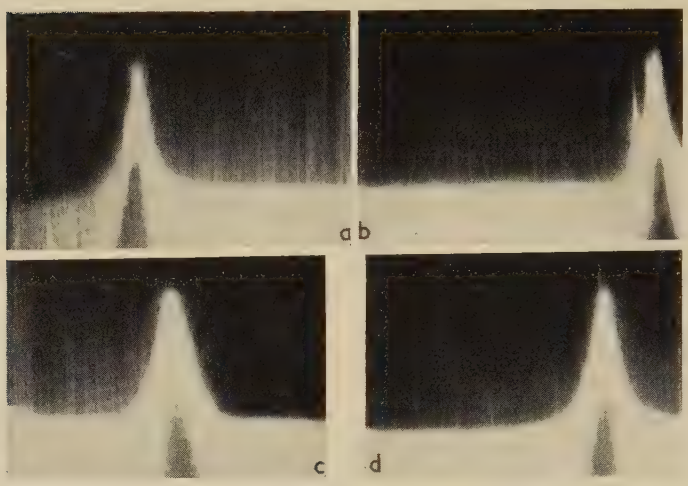

Fig. 1. Electrophoretic Patterns of the Lipoprotein: (a) ascending pattern in 0.1 M sodium citrate, pH 7.5, after 3630 seconds, field strength 18.0 volts/cm, (b) same, descending pattern, (c) ascending pattern in 0.1 M sodium acetate buffer, pH 7.8, after 2280 seconds, field strength 18.6 volts/cm, (d) same, descending pattern.

TABLE I

Analysis of the Protein Part of the Lipoprotein

	Percentage	Method
Nitrogen	14.5	Micro-Kjeldahl
Phosphorus	0.07	Allen (1)
Arginine	5.7	Thomas, Ingalls and Luck (20)
Tyrosine	3.2	Thomas (19)
Tryptophane	2.9	Spies and Chambers (15)

but the supernatant solution after the first precipitation showed a small absorption peak at 256 mμ.

A small residue remained after the extraction of the lipoprotein, but no "ghost" nuclei could be seen in this residue. It contained only small fragments. The lipoprotein was found to constitute roughly 50 per cent of the dry weight of the nuclei isolated by the Dounce method.

The nature of the lipoprotein. Extraction with hot alcohol in a Soxhlet apparatus removed lipid from the purified lipoprotein. The lipid constituted about 10 per cent of the total complex and gave positive tests for phospholipid and cholesterol. After the lipoprotein is purified as described above, no appreciable amount of lipid was removed during several more repre-

cipitations. Ether did not extract an appreciable quantity of the lipid from the moist precipitate of the lipoprotein nor from its alkaline solution.

Table I shows the results of analysis of the protein part of the lipoprotein (after removal of the lipid portion). Two-dimensional filter paper chromotography of an acid hydrolysate of the lipoprotein gave a pattern very similar to that of many other tissue proteins. The following amino acids were demonstrated to be present: alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, hydroxyproline, leucine (and isoleucine), lysine, methionine, proline, serine, threonine, tyrosine, valine.

Electrophoresis was carried out on the purified lipoprotein, using a Perkin-Elmer electrophoresis apparatus. The results obtained indicate that only one component is present in the region of pH 7.5.

Preliminary inorganic analysis indicates that the purified lipoprotein complex contains approximately 0.8 per cent calcium and no sodium or potassium.

DISCUSSION

It would be of interest to know what morphological part or parts of the nucleus contain the lipoprotein. The answer to this question is uncertain on the basis of evidence available at present. The lipoprotein fraction is essentially the same substance that Stedman and Stedman (16) called "chromosomin." The "residual chromosomes" of Mirsky and Ris (14), as prepared in our laboratory, were made up almost entirely of this same material (9). Nevertheless, it seems that the possibility of the nuclear membrane being made up of a lipoprotein complex should be given serious consideration. Only meager evidence has been obtained for that view, viz., the "ghost" appearance of many nuclei after extraction of the nucleohistone (referred to above) and the lack of any "ghost nuclei" after the extraction of the lipoprotein. We have not made careful microscopic observations to test this point, however. These observations were made incidentally during the progress of the chemical work and they are not new. Miescher (13) made some similar observations long ago. Bensley and Hoerr (5) and Bensley (3, 4) also reported somewhat similar findings.

SUMMARY

A lipoprotein, soluble in alkali and insoluble in water and neutral salt solutions, has been prepared from rat liver nuclei isolated by the Dounce and Behrens methods.

Hot alcohol extracted the lipid from the lipoprotein, but ether did not extract an appreciable quantity of lipid. Repeatedly dissolving the lipoprotein in alkali and reprecipitating with acid did not remove an appreciable amount of lipid. Tests on the lipid portion indicated the presence of phospholipid and cholesterol.

The amino acid content of the protein part of the complex was found by chromatography to be similar to that of many tissue proteins. It was found by chemical analysis to contain: nitrogen — 14.5 per cent, phosphorus — 0.07 per cent, arginine — 5.7 per cent, tyrosine — 3.2 per cent, and tryptophane — 2.9 per cent.

Electrophoretic patterns indicate that the lipoprotein complex contains only one component in the region of pH 7.5.

The possibility of the nuclear membrane being composed of a lipoprotein complex is discussed briefly.

We are indebted to Dr. Eugene Roberts of the Cancer Research Division, Washington University Medical School, St. Louis, Missouri, for carrying out the chromatography and to Dr. Charles W. Turner of the Dairy Husbandry Department, University of Missouri, who generously allowed us to use the Tiselius apparatus.

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CYTOLOGIC CHANGES IN THE SPERMATOGONIAL NUCLEI CORRELATED WITH INCREASED RADIO-RESISTANCE[1]

L. C. FOGG[2], and R. F. COWING[3]

Massachusetts Department of Public Health, Pondville Hospital, Walpole, Mass.[4]

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This study deals with the problem of identifying a morphological change specifically associated with increased radio-resistance in a primitive cell. Evidence can now be presented which suggests that certain morphological changes in the nuclei of the spermatogonia of the mouse are associated with increased radio-resistance.

It is generally recognized that cells in mitosis or cells in preparation for mitosis are radio-sensitive. Exceptions to this rule, however, are cited in the literature, as for example, Warren (6) and Warren and Dunlap (7) in a report on Effect of Radiation on Normal Tissue, emphasize that lymphocytes are more sensitive than other blood elements and this cannot be entirely explained on the basis of frequency of mitoses. Lacassagne (4) reported that radio-resistance among follicular cells of the ovary of the rabbit was not wholly a function of the primitiveness of the cell but rather a cytological state at the time of irradiation. Bloom (1) did not limit sensitivity to mitotic phases, and notes that meiotic spermatocytes are apparently more resistant to radiation than mitotic spermatogonia. Thus the question of the relation of differentiation to sensitivity even in reproductive cells is raised.

The germ cells in the mouse testis provide an opportunity to investigate this general subject of correlation of radio-resistance with nuclear differentiation. The spermatogonia either increase their kind by mitosis or differentiate into spermatocytes. Thus if there is a measurable difference in increased radio-resistance of a differentiating cell it should be demonstrable in a

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[1] With the collaboration of Miss Kathleen McCarthy, Junior Bacteriologist, Mass. Dept. of Public Health, Pondville Hospital.

[2] Chief of Research Laboratory, Pondville Hospital, Instructor in Pathology, Harvard University.

[3] Medical Nuclear Physicist, Cancer Research Institute, New England Deaconess Hospital, Boston, Massachusetts.

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spermatogonium evolving into a spermatocyte. An indication that this was the case was previously reported (2). In that report it was noted that there was a phase of the nucleated spermatogonia which appeared to have the typical reticulum of a prophase, but that these cells were not as sensitive within the period of twenty-four hours post-irradition as mitotic or even resting spermatogonia.

We recognize that cells which have been commonly called spermatogonia actually reveal extensive morphological variation. On study these variations assume a definite significance with respect to their roles either as cells in early mitosis or in early differentiation. This report is a cytologic study of the nucleus of the spermatogonium in an effort to correlate morphologic changes with the previously reported differences in radio-sensitivity of the spermatogonial nucleus within the period 24 hours post-irradiation.

It is proposed to show that there is an orderly series of changes in the direction of reproduction and another series in the direction of differentiation. It is further suggested that what appeared to be a spermatogonial prophase is only one step in the second series, thereby removing this prophase-like cell from the category of a true prophase.

MATERIALS AND METHODS

Testes of young adult pure line C57 black mice were used exclusively for the study. Testes, immediately after removal, were placed in Zenker's solution. Serial sections of tissue were stained in Heidenhain's iron hematoxylin or in hematoxylin and eosin.

The cytologic study constituted a survey of the germ cells of some fifty untreated or normal testes. The morphologic variations noted among the spermatogonial nuclei were evaluated and classified in the sequence of mitotic changes and the sequence evolving into spermatocytes.

OBSERVATIONS

Figs. 1–5 and Figs. 6–10 illustrate the orderly series of cytological changes that take place. It can be seen that not all nucleated spermatogonia which have been previously classified as resting cells properly fall into this category.

The truly resting spermatogonium (Fig. 1) has a nucleus composed of an evenly distributed, very finely granular chromatin mesh-work with several large, darkly staining nucleoli of varying sizes, a faintly basophilic nucleoplasm and a well-defined nuclear membrane. From this stem nucleus morphological variants occur which can be readily confused with it but

study reveals that early irreversible changes, either toward mitosis or toward differentiation into spermatocytes, are already established. The direction of these changes is recognized by the presence or absence of a thread-like arrangement of the still finely-granular chromatin.

Mitotic Series. Fig. 2 shows what is considered to be an early development toward reproduction. It is characterized by loss of a specific spireme, and the fact that the granular chromatin is assuming the form of compact bodies. These chromatin particles have a tendency to orient themselves peripherally giving an appearance of attachment to the nuclear membrane. This is sharply reminiscent of the "crust-like" nuclei as described by Bloom (1). Nucleoli are still present. Except for the factors mentioned, the nucleus resembles in size, shape and staining capacity its predecessor, the resting spermatogonial nucleus.

Fig. 3 shows a progression. The chromatin clumps have increased in number but there is no indication of their increase in size. The process is to continue until definitive chromosomes are formed. Fig. 3, therefore, represents only a transient phase. The orientation of the chromatin clumps tends to be peripheral and stays that way until the nuclear membrane begins to break down in late prophasic activity. In this figure the nuclear membrane is somewhat indefinite in outline and does not stain with clarity. At about this time, some suggestive evidences of early mitotic activity become apparent, such as, for example, the slight increase in size of the nucleus and the loss of staining capacity of the nucleoplasm. Fig. 4 and 5 illustrate a subsequent change typical of a phase of spermatogonial mitotic activity.

It may be emphasized here that quantitative data as previously reported (3) indicate that premitotic, mitotic and post-mitotic changes represent the stages which first react to ionizing radiation (1 through 24 hours). It was reported that the resting spermatogonia were sensitive also but that the effect did not become apparent until 20–24 hours post-irradiation. Progressive increased sensitivity is indicated therefore in the sequence from resting spermatogonial nuclei to mitosis. In the non-reproductive series increased resistance to ionizing radiation is noted.

Differentiating series. Fig. 6 illustrates one of the first phases in the direction of differentiation. It is evident that, in appearance, this phase and some of the subsequent nuclear developments may be easily confused with the resting spermatogonium, or with nuclei in early changes of the mitotic series.

The significant morphological difference between Fig. 6 and the resting spermatogonium is the accentuation of the reticulum. The reticulum, or the thread-like formation on which are arranged chromatin particles, is clearly

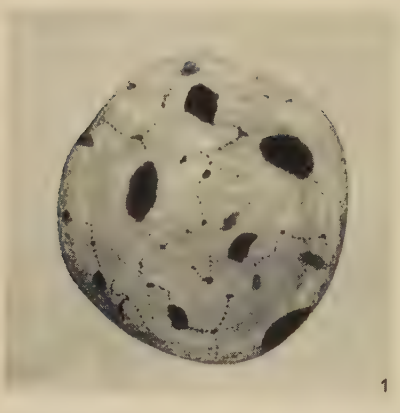
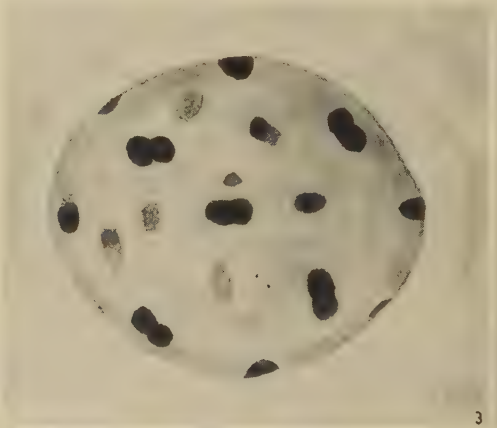
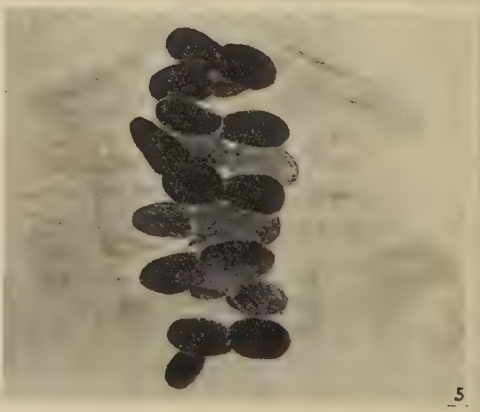


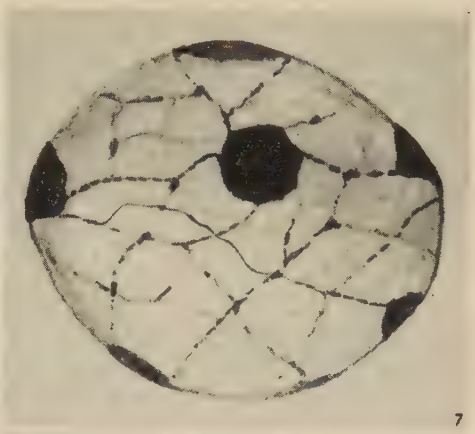
Fig. 1. Spermatogonial resting nucleus.

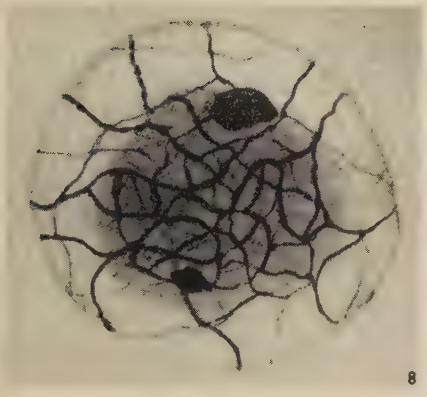
Mitotic Series:

Fig. 2. Early reproductive phase.
Fig. 3. Early prophase.
Fig. 4. Late prophase.
Fig. 5. Metaphase.



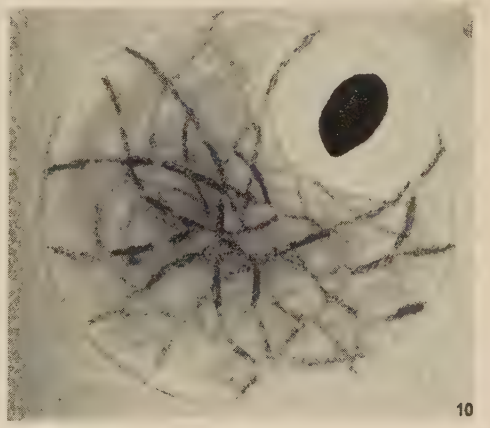






Differentiating Series:

Fig. 6. Early differentiation.
Fig. 7. Early differentiation showing prominent reticulum.
Fig. 8. Early differentiation showing orientation of reticulum.
Fig. 9. Prophase-like stage.
Fig. 10. Early spermatocyte.



Each plate is a pictorial representation of a selected nucleus as seen microscopically (970 ×).

apparent. The size and shape of the nucleolus, the well-defined nuclear membrane, and the staining capacity of the nucleoplasm are strikingly similar to the resting spermatogonium.

Fig. 7 represents a slightly later phase in nuclear differentiation. It is characterized by a still more prominent linin network, slight increase in size, a reduction in the number of large-size chromatin staining particles, the beginning of a lack of morphological integrity of the nucleolus, and a decrease in the affinity for stain of the nucleoplasm.

Fig. 8 shows a still later stage which further accentuates the increase in size of the reticulum and the gradual loss of the nucleolus. The nucleus is highly variable in size and shape. It would appear that while the reticulum has been uniformly distributed originally it is now being subjected to factors upsetting its orientation. In this figure, the reticulum is being reoriented toward the center, leaving the peripheral area faintly staining and sparse in solid stainable substance. At this stage there are many minor variants in size, shape and staining capacity. The nuclear membrane remains intact.

Fig. 9 represents a key phase, when considered from the aspect of its frequency of appearance as based on our quantitative method. It may be recalled that this nucleus is seen as frequently as or even slightly more often than a resting spermatogonial nucleus. It appears on noncritical examination very similar to the usual description of the late prophase. It has been described as a prophase-like or reticulated stage (2) but one which did not show the radio-sensitivity exhibited by the resting spermatogonial nuclei or by spermatogonial mitoses.

This nucleus is characterized by having a thick reticulum more or less oriented in a mass, and by the absence of a demonstrable nucleolus. The massed chromatin often is seen to be located at one side leaving a clear, non-staining nucleoplasmic area. A faintly staining but intact nuclear membrane can always be identified. Some increase in size is noted although this factor is variable.

Fig. 10 represents a phase clearly defined as an early spermatocyte. The enlarged reticulum is beginning to break up into units. A striking feature is the reappearance of a nucleolus, surrounded by a characteristic clear area. It can be seen that the nuclear membrane is intact. It may be emphasized here that the nuclear membrane never does disappear at any stage of the series. Growth has begun to take place, as shown by increase in size. This process continues until the mature primary spermatocyte has been formed.

All the nuclear changes described in both the mitotic and the differentiating series are summarized in Table I.

TABLE I

Spermatogonial Nuclear Cytology. Resting Nucleus.

	Linin Network	Chromatin	Nucleolus	Nuclear Size	Nucleoplasmic Staining	Nuclear Membrane
Fig. 1	Present	Scattered Masses	Present	Typical	Basophilic	Distinct
			Mitotic Series.			
» 2	None	Scattered Masses	Present	No Change	Faintly Basophilic	Distinct
» 3	None	Organizational Masses	Indistinct	No Change	Indeterminate	Intact Faintly Staining
» 4	None	Chromosomal	Absent	Enlarged	A-chromatic	Absent
			Differentiating Series.			
» 6	Present Fine Threads	Scattered Masses	Present	No Change	Basophilic	Distinct
» 7	Present Enlarged	Oriented on Linin Network	Present	No Change	Basophilic	Distinct
» 8	Present Prominent	Linin and Chromatin — Now a Reticulum	Present Indefinite	Variable, Generally larger	Faintly Basophilic	Distinct
» 9	Reticulated and like	Prophase-	Disappeared	Slightly	A-chromatic	Intact but Irregular
» 10	Less Compact	Reticulum	Nascent	Slightly Enlarged	A-chromatic	Intact

DISCUSSION

The observations recorded here have been made in an attempt to clarify spermatogonial nuclear cytology. Our evaluations are based on a review of fifty non-irradiated C57 black mice. Selected slides, representative of the available material, were utilized for detailed examination.

The utilization of cytological variations in the nucleated spermatogonia arranged in an orderly sequence toward reproduction or toward differentiation contributes to the identification of the various types of spermatogonia. While the variants referred to, in general, may be classified simply as spermatogonia the need for greater precision of terminology is evident when special problems, such as that of radio-sensitivity, are being considered. The truly resting spermatogonium appears to be seen in one form only, as illustrated in Fig. 1, and probably corresponds to the type of cell sometimes described as "dust-like." The reported prespermatogonia, "crust-like" nuclei, and the large or small or darkly staining cells are present. These terms are merely descriptive, however, and give no hint of the special activity, either mitotic or differentiating, inherent in the cells being described. Preliminary observations of spermatogonial nuclei in rats (pure line Sloniker strain) and in some human testes suggest the same orderly spermiogenesis as described for mice.

The knowledge of the cytology can be of practical use to the histopathologist in determining early changes or effects resulting from the application of ionizing radiations. The primitive spermatogonial cell, commonly called embryonal, and the differentiating one can be detected with certainty. A casual examination of spermatogonial nuclei as seen in a cross-section of a tubule of a testis reveals two clearly apparent variants, one the typical resting and the other the reticulated or prophase-like nucleus. The latter occurs at a frequency equal to or higher than the resting nucleus. It is not as sensitive to irradiation as prophases are generally supposed to be. The demonstration that this nucleus is not in a true prophase, therefore, further confirms the belief that a prophase is highly sensitive to ionizing radiation, while differentiation is usually accompanied by comparatively greater resistance.

Since the reticulated nucleus represents an irreversible step toward differentiation it is not clear why mitotic stages are seen so infrequently. A reference to frequency tables (2) shows that of each one hundred tubules examined as many as fourteen may show presence of mitoses. It is difficult to reconcile this figure with the millions of sperm which are produced periodically. The

matter deserves further investigation. The possiblility of eliminating definitely the differentiating nucleus may make it possible to obtain more critical quantitative data on this problem.

SUMMARY

The morphological variants in nucleated spermatogonia are described. Only one form of resting nucleus is recognized. The other variations can be arranged in an orderly sequence in the direction of spermatogonial reproduction or of differentiation.

Reproductive nuclei are characterized by loss of reticulum. Differentiation in the direction of spermatocyte formation is recognized by retention of reticulum.

It is suggested that the reproductive series is increasingly radio-sensitive, while the differentiating series is more resistant. This confirms the observation that the frequently appearing reticulated nucleus is more resistant than a mitotic or resting spermatogonial nucleus.

It has been demonstrated that recognition of increased radio-resistance in an early differentiating nucleus is possible by a knowledge of its cytology.

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THE ROLE OF THE JELLY COAT IN THE UPTAKE OF CALCIUM BY EGGS OF *ARBACIA PUNCTULATA* BEFORE AND AFTER FERTILIZATION

F. H. N. RUDENBERG[1]

Department of Physiology, University of Chicago, Chicago, Ill., and Marine Biological Laboratory, Woods Hole, Mass., U.S.A.

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There is an extensive literature on the importance of calcium in the sea urchin egg with respect to calcium being essential for the interblastomere cement substance (8), for fertilization (16), for activation (26), for the surface precipitation reaction (6), and for the maintenance of the semipermeable plasma membrane (7). Changes, on fertilization, of the amount of free calcium within the egg were noted by Mazia (18) and of total calcium by Örström and Örström (22) and Monroy-Oddo (21); however, Lindvall and Carsjö (15) noted no change in total calcium. Lansing and Rosenthal (10, 12) believe that calcium is associated in the cortex with ribonucleic acid and Monroy (20) noted changes in cortical birefringence which he ascribes to changes in calcium and potassium bonding of lipoproteins.

Before considering the possible roles which calcium might play in such changes in cellular ultrastructure as may occur at fertilization and subsequent cell divisions, it would seem necessary to ascertain whether sea urchin eggs do, in fact, take up calcium from their environment during the early stages of development. In the experiments reported here the uptake of calcium was followed continuously by the disappearance of radioactivity from the medium. It will be shown that the eggs first take up and then lose calcium. Evidence is presented that these changes do not involve the protoplasm but must, instead, be ascribed to the jelly.

MATERIALS AND METHODS

Preparation of the Gametes. Eggs and sperm of adult sea urchins, *Arbacia punctulata*, were obtained by removal of the gonads from dissected animals. Sperm was maintained in the "dry" state until use. Eggs were filtered through cheese cloth and

[1] Atomic Energy Commission Predoctoral Fellow in the Biological Sciences, 1950–51. Part of a dissertation submitted to the Department of Physiology, University of Chicago, for the degree of Master of Science.

washed repeatedly in twice-filtered sea water. In some experiments the jelly coats were removed by gentle shaking. The eggs were subsequently concentrated by settling and were added rapidly to the incubation medium. Fertilization was effected in the usual manner.

Technique. Preliminary observations were carried out to determine the rate of settling of *Arbacia* eggs and embryos. All stages settled more than one millimeter in ten seconds with jelly coats, and faster without them. Beta particles emanating from radioactive calcium, Ca^{45} (maximum energy 0.26 Mev), are completely absorbed in 0.8 mm of water (infinite thickness). Thus, as soon as the eggs had settled this distance any radioactivity emanating from the eggs was absorbed in the medium and none reached the Geiger tube. Since the dish was stirred thoroughly, the radioactivity measured represented a true sample of the radioactivity of the medium around the eggs. Thus if the eggs took up some radioactive calcium, the radioactivity of the sea water decreased; this decrease could be measured because the technique was made sufficiently sensitive by the use of large numbers of eggs in a minimal volume. For plotting the curves, however, since one is interested in the movement of Ca^{45} with respect to the egg, the ordinate was plotted so as to decrease in counts up the *y*-axis; in this way a positive slope represents an increase in egg Ca^{45}.

Apparatus. A constant temperature bath ($20.0 \pm 0.1°$ C.) was placed in an air conditioned room (21° C.) used for measuring radioactivity. A tray in the bath supported two six-inch petri dishes. These were covered by fitted lucite plates through which glass stirrers projected. One dish contained experimental eggs and the other control eggs. A hole was cut in the cover of the experimental dish to hold and support a lead-shielded thin window Geiger tube just above the medium. Scaler readings of the number of counts were taken for one minute every second minute. The stirrer then was turned on for half a minute and a further half minute elapsed before the next reading. Some experiments were carried out at a higher temperature under similar, but not identical, conditions.

Incubation Medium. In order to obtain a scaler count of about 2 500 disintegrations per minute (expected error 1.5 per cent), enough radioactive calcium (1.05×10^{-2} mg or 0.52 μc per ml Ca^{45}) was added to twice filtered sea water to give this rate. The radioactivity did not affect the eggs in any observable way. Varying volumes of this radioactive sea water were placed in the experimental dish and a similar volume of identical, calcium enriched, but not radioactive sea water was placed in the control dish. Two additional control dishes were left at room temperature and were agitated by hand; neither of these was radioactive, one being calcium enriched sea water and the other normal twice-filtered sea water. All four dishes had a pH of normal surface sea water, pH 8.2–8.4, and there was no change during an experiment (except in Experiments 3 and 4). No buffers were used because the experiments were intended to determine the rate of uptake of calcium under conditions as close to those in nature as possible.

As soon as the measurement of radioactivity emanating from radioactive sea water reached a constant rate of counting, eggs were added to each dish. The experimental eggs were crowded to a maximum of 3.5×10^6 per 10 ml and the control eggs in the bath were more dilute (1×10^3 per 10 ml). However, both developed to the pluteus stage at the same rate. A slightly faster rate of development of the controls at room temperature permitted prediction of the time of cleavage of the

TABLE I

Representative Experimental Conditions.

I Experiment	II Remarks	III Counts per Minute Before Adding Eggs	IV Counts per Minute on Adding Eggs	V Ml. Settled Eggs	VI Number of Eggs $\times 10^6$	VII Total Volume Before Fertilization Ml	VIII Hours Unfertilized	IX Hours Fertilized
1	Jelly Coats Intact 26° C. pH Unknown	—	2 350	1.17[1]	1.7	64.5	0	6
2	Jelly Coats Intact 26° C. pH Unknown	—	2 270	1.17[1]	1.7	64.5	0	6
3	Jelly Coats Intact 25.5° C. pH 7.6	—	2 540	2.07[1]	3	67.5	0.25	8.25
4	Jelly Coats Intact 25.5° C. pH 7.6	—	2 480	2.07[1]	3	67.5	0.25	8.25
5	Jelly Coats Intact	2 120	1 700	5	7.3[1]	126	3.5	8.5
6	Jelly Coats Intact	3 770	2 800[α]	10	14.5[1]	70	2	9.5
7	Jelly Coats Washed Away	3 050	2 180[α]	10	30.0[1]	85	4.5	11
8	Jelly Coats in Solution	—	1 920	6	21.0[1]	50	5	10
9	Jelly Coats Shaken off with time	—	2 470	3	4.3[1]	10	3.4	2.2
10	Jelly Coats Shaken off with time	—	2 100[β]	1	1.5[1]	15	4.1	4.1

α Unfertilized eggs subsequently corrected for dilution at fertilization for Fig. 1 and 2.
β Fertilized eggs subsequently corrected for dilution at fertilization for Fig. 3.

eggs in the bath. Fertilization was effected after studying the unfertilized eggs for several hours. All control dishes were examined periodically. Regulations regarding the use of radioactive liquids in the scaler room prevented frequent observation of the experimental dish; however, at the end of the experiment the embryos in this dish had developed as far as those in the control dish. All dishes were examined for damaged eggs, for the presence or absence of the jelly coat (with india ink particles), and for rate of development. In addition, the period of cleavage was followed carefully for the first four divisions. If fertilization was not 98 per cent or better or if embryos were not viable to this extent the experiment was discarded. Experiments were continued to the time of hatching of the swimming blastula — 10 hours from fertilization at 20.0° C.

An indirect method was used because there was a shortage of *Arbacia*, because in this way one batch of eggs could be studied continuously during their develop-

[1] Estimated value.

ment, and because washing eggs preliminary to determining their radioactivity introduces errors.

The experimental conditions of representative experiments are listed in Table I. In Experiments 5 to 10 the temperature was maintained at 20.0° C. and the pH was 8.2.

RESULTS

The results of representative experiments are listed in columns 2 and 3 of Table II. These results are averages read from curves plotted from the experimental data. Fig. 1, 2, and 3 are representative curves of the different types of experiment. They are plotted as described above so as to appear as if the eggs are being observed. In each case a correction has been applied (see Table I) for the dilution at fertilization due to the addition of dilute sperm (except in Table III, for example).

The observations (25) show that eggs which are surrounded by jelly coats take up Ca^{45} both before fertilization and after fertilization for about six hours (to the 256 cell stage) and then begin to lose it, presumably coincident in timing with the dissolution of the jelly coat by an egg secretion (Fig. 1). If eggs have their jelly coats removed before an experiment no uptake of Ca^{45} may be observed (Fig. 2). This is also true if the jelly coats are removed from the eggs but left in solution in the radioactive medium. Toward the end of the season the jelly coats come off eggs rather easily. Thus in some experiments (Fig. 3) eggs were incubated in radioactive medium for some time, before an experiment was started. As the experiment progressed the jelly coats came off the eggs, and more radioactivity emanated from the medium indicating that the two observations may be related.

Since the counting technique, as shown by measurements of a radioactive standard or of radioactive sea water, is accurate to only $\pm$ 5 per cent, no variations in calcium movement such as shown by Monroy-Oddo (21) are discernible. Statistical analyses of Experiments 6 and 7 have been carried out and are listed in Table III. The fertilized eggs were analyzed for only the first few hours, since during only this time are the cleavages in synchrony. Analyses of each group of five determinations, i.e. each ten minute period, indicate a varying standard deviation of the order of that listed in Table III and within the limits of the variations introduced by the radioactive scaler.

In order to understand more clearly the significance of the data, certain calculations are listed in the last three columns of Table II. These are based on the following assumptions: (a) the egg has a diameter of 74 μ and is surrounded by an 18 μ thick jelly coat (measured from photographs), (b)

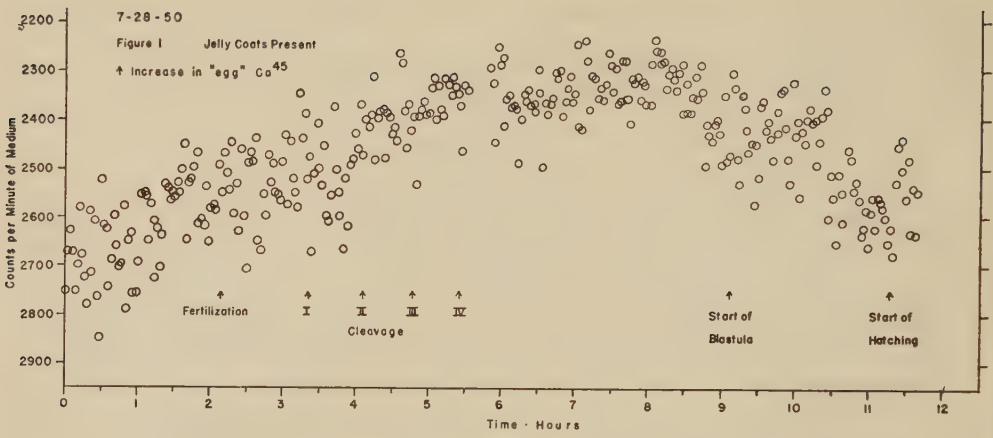

Fig. 1. Uptake and loss of radioactive calcium by sea urchin eggs with jelly coats. Experiment 6.

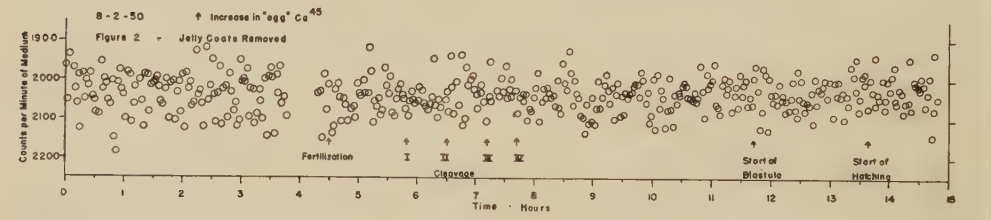

Fig. 2. Absence of uptake and loss of radioactive calcium by sea urchin eggs with jelly coats removed. Experiment 7.

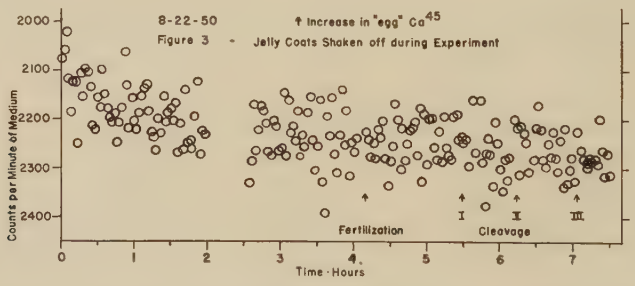

Fig. 3. Loss of radioactive calcium (coincident with shaking) by sea urchin eggs after incubation. Experiment 10.

the egg contains 0.3 mg Ca per ml eggs (18), and (c) sea water contains 0.35 mg Ca per ml as found by the titration of oxalate by permanganate. Thus one ml contains 3.49×10^6 eggs without jelly (74 per cent of space) or 1.45×10^6 eggs with jelly.

It must be realised that variations exist among the experiments and hence from the calculations only orders of magnitude are of value. Thus, embryos developing at about 26° C. seem to show an increase of around 0.3 mg Ca per hour per 10^6 embryos while those at 20° C. show about one quarter that rate. The warmer temperature approximately doubles the rate of jelly coat dissolution occurring at 20° C. The rate of disruption by shaking is lower than the rate of dissolution.

DISCUSSION

The striking difference in calcium uptake by eggs with and without jelly coats indicates that eggs immersed in a "physiological" medium with a slight excess of calcium do not accumulate any calcium in the absence of the jelly coat. Although measurements during the dilution of the radioactive solution at the time of adding the eggs do not show whether or not the egg is permeable to calcium, they do show that the egg does not accumulate calcium rapidly or to any extent. This was also shown by direct measurements of the radioactivity of a washed concentrate of eggs. The absence of a change in the slope of the curves at the time of fertilization indicates that there is no change in the rate of uptake at this time. Since the rate of development is the same both in the presence or in the absence of the jelly coat and since, as indicated in Fig. 3 (Experiment 10), Ca^{45} reappears in the incubation medium when eggs are shaken gently (late in the season) to disrupt their jelly coats, it may be concluded either that the jelly coat of the sea urchin egg begins to take up calcium as soon as the egg is immersed in sea water or that the calcium in the jelly coat begins to exchange with that of the medium. Since chemical analyses for total calcium have not yet been carried out, one may conclude only that one or the other of these two possibilities exists. However, if an exchange of Ca^{45} is occurring, rather than an accumulation, then the calcium in the jelly coat either has fully exchanged within fifteen minutes or it has not fully exchanged before the time that the egg begins to secrete the hatching enzyme. Such a hatching enzyme has been shown in *Arbacia* by Kopac (9).

From the data in Table II it may be noted that the Ca^{45} uptake each hour varies from an average of one times (20° C.) to over three times (25–26° C.) the egg content established by Mazia (18) and the ratio with the exchangeable calcium per cell (19.2 ± 10^{-9} mg) is even greater (19). These calculations thus serve as a further indication that calcium is being accumulated in the jelly coat and that the egg does not accumulate calcium. Other calculations

TABLE II

Representative Experimental Results and Calculations.

I	II	III	IV	V	VI
Experiment	Period During which Change Occurred Hours from Egg Addition	Change in Counts per Minute per Hour per 10^6 Embryos	Total Calcium in Medium Mg	Mg Calcium per 100 Counts per Minute After Egg Addition	Change in Mg Calcium per Hour per 10^6 Embryos
1	0 to 6	+29.4	24.7	1.05	+0.309
2	0 to 6	+35.2	24.7	1.09	+0.384
3	0 to 5.5	+36.4	25.2	0.99	+0.360
	5.5 to 8.5	−20			−0.198
4	0 to 6	+28.4	25.2	1.02	+0.290
	6 to 8.5	−32			−0.326
5	0 to 9.5	+ 5.2	43.2	2.54	+0.132
	9.5 to 12	− 7.1			−0.180
6	0 to 5.5	+ 4.8	22.6	0.81	+0.039
	5.5 to 8.5	0			0
	8.5 to 11.5	− 6.9			−0.056
7	0 to 15.5	0	28.6	1.31	0
8	0 to 15	0	16.8	0.87	0
9	0 to 1	−20.9	2.7	0.11	−0.023
	1 to 5.6	0			0
10	0 to 2.2	−39.4	5.2	0.25	−0.098
	2.2 to 8.2	− 3.3			−0.008

show that a change in Ca^{45} content of the eggs of about 5 per cent per hour for five hours would be detectable by the techniques employed.

Although oscillations as have been described by Brooks (2) are not visible because of the $\pm$ 5 per cent variations of the determinations, these are unlikely since the variations at the time of egg addition (when oscillations might be expected) were statistically no greater than at other times during each experiment. Since all points on Fig. 1 and 2 (and 3) fall within approximately $\pm$5 per cent of the median and since the standard deviations are all of the order of those of the radioactive standard, the fluctuations, with time, of the counting rate are not significant. Therefore the possibility of cycles of calcium uptake and loss correlated with changes in ultrastructure is neither indicated nor excluded.

Whereas Warburg (32) has shown that the metabolism of sea urchin eggs

TABLE III

A. Statistical Analysis of Parts of Fig. 1, Experiment 6 (7–28–50), and of Radioactive Source used as Standard.

	Mean Counts per Minute	Standard Deviation	Standard Error of Mean	Duration of Analysis Hours
Unfertilized Eggs	2 794 to 2 658[1]	±58	±7.3	2
Fertilized Eggs	2 561 to 2 314[1]	60	5.7	4
Standard	2 093	78	2.4	1

B. Statistical Analysis of Parts of Fig. 2, Experiment 7 (8–2–50), and of Radioactive Source used as Standard.

	Mean Counts per Minute	Standard Deviation	Standard Error of Mean	Duration of Analysis Hours
Unfertilized Eggs	2 184	±52	±4.7	4.5
Fertilized Eggs	2 041	43	3.5	5
Standard (8–3–50)	3 041	42	3.4	0.5

increases markedly at the time of fertilization and various investigators have shown that the permeability to water (17) and to certain radioactive salts increases greatly on fertilization (1, 3, 4, 5), there is no change in the rate of uptake of Ca^{45} by *Arbacia* eggs on fertilization. This suggests that there is no change in permeability to Ca^{45} or no change in accumulation. Furthermore, it is somewhat difficult to understand why there should be a loss of calcium from the sixth hour after fertilization, should this represent a loss of calcium from the egg protoplasm, since one might expect the egg to take in calcium preparatory to the start of spicule formation which occurs soon after the termination of these experiments. Although differences in the length of study before fertilization occurred, it should be noted that in experiments with intact jelly coats, the change in slope observed some hours after fertilization consistently occurs at the same stage of embryonic development. This indicates that the hatching enzyme already is secreted by the 256 cell stage.

The presence of calcium in the jelly coat was first indicated by R. S.

[1] A mean was determined for each ten minute period of the analysis and the mean for the first and for the last ten minute period is given.

Lillie (14). Further analysis by Vasseur (30) showed the jelly coat to be composed of 75–80 per cent polysaccharide esterified to sulfate and 20–25 per cent protein. He also found (31) that 70–90 per cent of the cations are calcium and that calcium makes up about 8 per cent of the total weight. The jelly coat has been of interest since F. R. Lillie showed it to contain a sperm activating, agglutinating, and chemotactically influencing substance (13). Tyler, in his investigations of the gamones (28), reaffirmed the dependence on calcium of the fertilizin-antifertilizin reaction (29).

Mazia (18) found that although the total calcium content of the *Arbacia* egg remains constant on fertilization, the proportion of bound calcium decreases by about 15 per cent. Örström and Örström (22) reported that *Paracentrotus* eggs lose calcium on fertilization and that the content then remains constant until spicule formation, and Monroy-Oddo (21), studying the Naples form of *Arbacia*, found a loss of calcium after fertilization with a subsequent increase during the second half hour after fertilization. On the other hand, Lindvall and Carsjö (15) noted the absence of a change in total calcium in jelly-free eggs of *Echinus*. The conclusions reported above are compatible with these findings.

Several other studies of the Ca^{45} uptake of living cells have been carried out (12, 23, 24, 27, 33). Of these, two concern a study of intact single cells. There is preliminary evidence (24) that *Paramecium* does take up Ca^{45} when incubated in a hay infusion and Woodward (33), although unable to get a "usable" amount of Ca^{45} into an isolated single muscle fiber, did show an uptake of Ca^{45} by whole frog sartorius muscles. Studies of the mechanism of uptake, accumulation, or exclusion of calcium thus seem to be imperative before one may ascribe any significance to the apparent lack of accumulation of calcium by the sea urchin egg reported here.

SUMMARY

1. The uptake of radioactive calcium from calcium-enriched sea water by the unfertilized and fertilized eggs and embryos of the sea urchin, *Arbacia punctulata*, has been investigated up to the time of the swimming blastula stage.

2. It was found that normal eggs surrounded by intact jelly coats take up radioactive calcium before as well as after fertilization until about six hours after fertilization when a loss of calcium to the medium is observed. This loss begins at about the 256 cell stage and is believed to be associated with the secretion of the hatching enzyme by the egg.

3. No change in the rate of increase of "egg" radioactivity has been noted on fertilization.

4. Eggs that have their jelly coats removed by gentle shaking, whether these are washed out of or remain in solution, show no uptake and no loss of calcium for several hours before fertilization as well as for the ten hours of development to the swimming blastula stage (20.0° C.).

5. Eggs incubated in radioactive medium and then studied as the jelly coats are disrupted and dissolved in the medium show a loss of "egg" radioactivity which is followed by a constant level of radioactivity emanating from the medium.

6. From this evidence and from some theoretical considerations it is concluded that the egg jelly coat accumulates calcium and that the *Arbacia* egg does not accumulate radioactive calcium during the period studied.

7. Statistical analyses neither indicate nor exclude the possibility of cycles of calcium uptake and loss correlated with cell division within the $\pm$ 5 per cent range of accuracy permitted by the technique.

The author is indebted to Dr. J. M. Tobias for his frequent and helpful advice during the preparation of the manuscript and to Dr. J. H. Welsh for directing the investigations (24) from which the present study developed. The possible importance of the jelly, suggested by Dr. J. Runnström, led to experiments with jelly coats removed, and Dr. E. B. Harvey provided sea urchins after the author's supply had been exhausted. Thanks are also due to the Directors and to Dr. G. Failla and Mr. N. Baily for facilities made available at the Marine Biological Laboratory.

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THE PROPERTIES AND EFFECTS OF OSMIUM TETROXIDE AS A TISSUE FIXATIVE WITH SPECIAL REFERENCE TO ITS USE FOR ELECTRON MICROSCOPY

K. R. PORTER and F. KALLMAN[1]

Laboratories of The Rockefeller Institute for Medical Research, New York, N.Y.

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It is remarkable that a compound as obscure as osmium tetroxide should have been found to be a good fixative relatively early in the history of histology. Baker (3) reports the earliest description of its use to have been presented in 1865 by Max Schultze in a study of the marine protozoan, *Noctiluca*. Subsequent expansion in the utilization of this compound for histological purposes is well known and it is now one of the essential components of classical fixatives which differentiate mitochondria, Golgi, fat, myelin, etc. The use of osmium tetroxide alone in fixation has been less extensive, due to its slow penetration and its interference with various classical staining methods.

Although nuclei are reportedly not well preserved by osmium tetroxide, it is generally agreed that OsO_4 is an excellent fixative of cytoplasmic structures, since it preserves the outlines and form of structures seen in living material, and does not cause a visible coagulation. Little is known, however, concerning the chemical action of OsO_4. Such studies as those of Hardy (14), Monckeberg and Bethe (18), Fischer (13), Mann (16), Hofmann (15), Berg (5), and Baker (3), have provided only rough indications of the reactions of various fixatives, including osmium tetroxide with proteins and fats.

That more precise chemical studies of fixation have not been made is due partly to the difficulty involved in assessing the quality of fixation. What criteria can be used to decide when a cell is well fixed? The visibility of a structure after fixation that was not apparent before, may simply mean that fixation has altered its refractive index relative to its surroundings. Hence, to label all such structures artefacts, is ill-considered. Probably the optimum requirements to be made are that the cell should not detectably change its shape under the action of the fixative and that its cytoplasm should not,

[1] Post-doctorate Fellow of the National Cancer Institute, National Institutes of Health, Federal Security Agency.

under high resolution, show discontinuities and lacunae of irregular size and angular form. Such structures with associated surfaces, and density differences, if present in the ground substance of the living cell, would most probably scatter light in dark field microscopy, whereas under such examination the ground substance is normally quite clear and nonrefractile.

Osmium tetroxide has recently come to be used in the preparation of tissue cells and other biological objects for electron microscopy. Here, even more than for light microscopy, it is essential to obtain faithful preservation of structures and to have a more extensive knowledge of the action of the fixing agent. Interest in the properties of the compound has therefore increased and has stimulated the present limited study. This report will review what is known of the compound, present evidence to demonstrate the perfection of its action as a fixative, and show its effects on tissue cells with time of action. It is hoped that the observations presented and the problems defined will elicit more critical analyses, especially of the chemical action of OsO_4 on biological materials.

Chemical Properties of OsO_4. Osmium, element 76, is a grey to white metal with an atomic weight of 190.2. Its density per unit volume is the highest of any known element. It is found associated with platinum as osmiridium in the Witwatersrand gold reef of South Africa, in the Ural mountains, and in Alaska. It is used to a limited extent commercially in hard alloys for tipping fountain pen nibs and phonograph needles, and as pivots for scientific instruments.

The tetroxide is the highest known oxide of osmium. It is formed by oxidizing the metal in solution with *aqua regia*, after which the volatile oxide is distilled off from the solution. In the solid state it consists of volatile white to yellowish crystals which melt at 41° C. The liquid boils at 131.2° C. It has a characteristic, slightly sweetish odor which can be detected to the limit of 2×10^{-5} mg per cc of air (12). The vapor is dangerous in that it attacks and fixes the moist cellular surfaces of the nose, throat, and eyes. When vapors of OsO_4 come in contact with the skin or other tissue surface, the area of contact turns dark brown to black. This is claimed by Thorpe and Whiteley to result from the formation of a hydrated dioxide of osmium, whereas Ephriam (12) states that the colored product is $Os(OH)_4$.

Osmium is known to exist in many valency states. Compounds belonging to the +2, +3, +4, +6, and +8 states have been described. The existence of osmium with a valence of +7 is in doubt, although an unstable halogen derivative of this nature has been described (10). Opinion differs as to the existence of various oxides and hydroxides. Thorpe and Whiteley (23) state that only the tetroxide and dioxide are definite compounds, although others claim the existence of OsO, Os_2O_3, $Os(OH)_3$, and $Os(OH)_4$. Of these, OsO and OsO_2 are reported to be grey to black, Os_2O_3 to be dark brown and gelatinous, $Os(OH)_3$ to be reddish brown, and $Os(OH)_4$ to be dark brown and gelatinous (12).

The value of osmium tetroxide as a fixing agent is apparently dependent upon its ability to act as a strong oxidizing agent and to affect various components of

living material differentially, as determined by their composition and reactivity. It is known to combine actively at ethylenic double bonds to form five membered rings (8, 9), and more recently it has been demonstrated to react selectively with certain double bonds in aromatic hydrocarbons (7, 2).

Osmium tetroxide is neutral to litmus and is a non-electrolyte. For this reason the practice of calling it "osmic acid" is misleading and incorrect. Osmium tetroxide can be quantitatively estimated colorimetrically by heating with hydrochloric acid and thiourea (19).

Action as a fixative of Cells. Osmium tetroxide in solution or as vapor is seldom applied as a fixative to blocks of tissue which are to be sectioned, although it is a frequent component of classical fixatives used for this purpose. That it has not been used by itself, stems from the experience that it penetrates the tissue slowly, gives uneven fixation and produces sections which tend to crumble. Moreover, many stains are ineffective after osmium fixation. Some of these ills can be overcome by applying it to the tissue as a perfusate (11), but this generally is not a convenient procedure.

For isolated cells such as protozoa or tissue units, pure OsO_4 is much more satisfactory. One of the earliest and most convincing demonstrations of this came from a study made by Strangeways and Canti (22), in which the action of various fixatives was compared on cells during examination in the dark field microscope. Of all the agents tried, OsO_4 produced the least detectable change. As the reagent reached the cell, the most evident change was an increase in light scattering by the nucleus. This describes, according to these authors, the development of a "fine precipitate."

These observations are readily confirmed. Tissue culture cells examined with the phase contrast microscope and under dark field illumination exhibit an instantaneous cessation of Brownian movement in the cytoplasm when exposed to the vapors of 2 per cent OsO_4. The cytoplasm retains the transparency of the living cell. In time, there is selective action on the cytoplasmic constituents as evidenced by gradual browning of fat droplets and increased definition of membraneous structures, but, initially, the predominant action of the treatment is one of gelation.

The electron microscope image of cells fixed with OsO_4 essentially bears out these light microscope findings. Evidence of this is provided in Fig. 1, which shows a rat myoblast which was fixed with OsO_4 vapors while bathed in a hanging drop of balanced salt solution (Earle's formula of Tyrode) at pH 7.4 (for methods see 20 and 21). The cell outline is that of the living unit and shows little evidence of shrinkage or contraction. The cytoplasmic surface and content are smooth (see also Fig. 5) with no aggregates or lacunae such as accompany coarse coagulation. Small arrays of fibers apparent in the

thinner margins of the cell resemble those which may be seen in phase contrast images of similar cells. The outlines of the formed structures of the cell such as nucleus and mitochondria are smooth and do not show the angularity of distortion expected in poor fixation. The entire image is descriptive of an extremely faithful preservation of native morphology.

The superior quality of this OsO_4 fixation is emphasized by comparing it with the action of other reagents or mixtures commonly used in tissue preservation. For example, Figs. 2 and 3 show the electron microscope image of portions of cells which had been fixed with Flemming's solution.[1] It is clear from the micrograph of the shadowed material especially, that the cell has suffered considerable damage. It appears that existing condensations of fibrous elements in the cytoplasm have served as centers for the coagulation of the otherwise homogeneous matrix with the concurrent development of interfibrillar lacunae. As mentioned earlier, such variations in density and distribution of non-volatile matter could not exist in the living unit and remain undetected by dark field or phase contrast microscopy.

It has been found that the character of the image varies with the time the material is subjected to the action of OsO_4 vapors. Nothing new in the nature of morphological entities is added to the cell. Instead, certain components are removed and the remaining structures are thereby revealed with greater clarity.

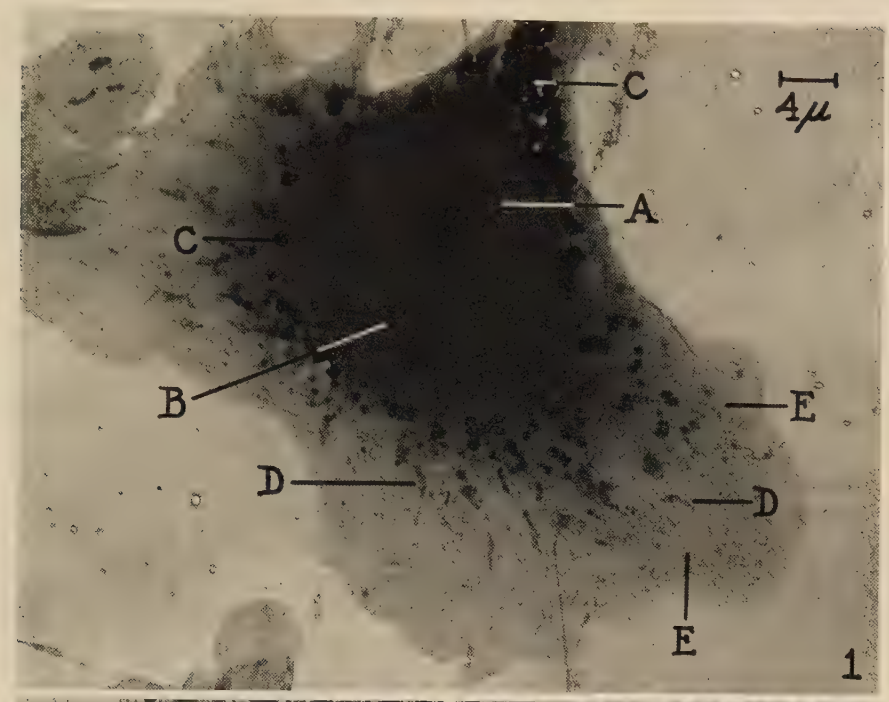
After a period of 10 minutes over vapors of OsO_4 (Figs. 4 and 5) the mitochondria (*A*), strands of the endoplasmic reticulum (*B*) and small fiber arrays (*C*) in the cytoplasmic matrix may be identified. It is evident, however,

[1] Flemming's solution (strong formula, i.e. with acetic acid) was chosen to exemplify the action of most cytological fixatives, all of which (except HCHO) are similar in causing some degree of coagulation in the cytoplasm. Unpublished studies of Dr. Bruno Schober have demonstrated that relatively coarse patterns of cytoplasmic structure are provoked by the action of all such common fixative constituents as potassium dichromate, mercuric bichloride, chromic acid, iodine, picric acid, and absolute alcohol.

Fig. 1. Electron micrograph of a fibrocyte grown in tissue culture from an explant of rat heart. The cell was fixed with vapors from 2 per cent OsO_4 for 10 minutes and, following washing and drying, was shadowed with chromium (for methods see 20 and 21). The natural outline, the absence of obvious shrinkage, and the smoothness of the cell surface define the fixation as extremely faithful. The nucleus is at *A*, the centrosome at *B*. The osmium dense lipid granules are at *C*, the mitochondria at *D*, and a small array of intracellular fibrils at *E*. The magnification, 1 800 ×, is not sufficient to depict other structures. This and all the micrographs on Plate III were taken with a Philips E.M. 100; all other figures were taken with an R.C.A. type E.M.U.

Fig. 2. Micrograph of portion of cell similar to that shown in Fig. 1 but fixed in Flemming's solution. The margin of the cell is at *A*. The dense strands and clumps and accompanying lacunae are in part a product of coagulation. Mag. 4 900 ×.

Fig. 3. Micrograph of a small part of the cytoplasm of a fibrocyte similarly fixed in Flemming's but here shadowed with chromium. The surface irregularities resulting from the destructive fixation are obvious. Mag. 15 000 ×.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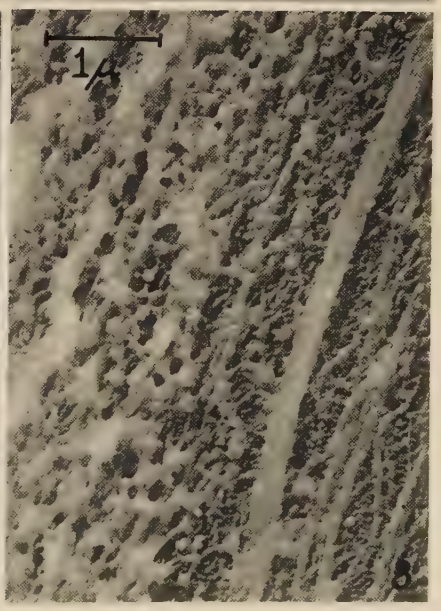
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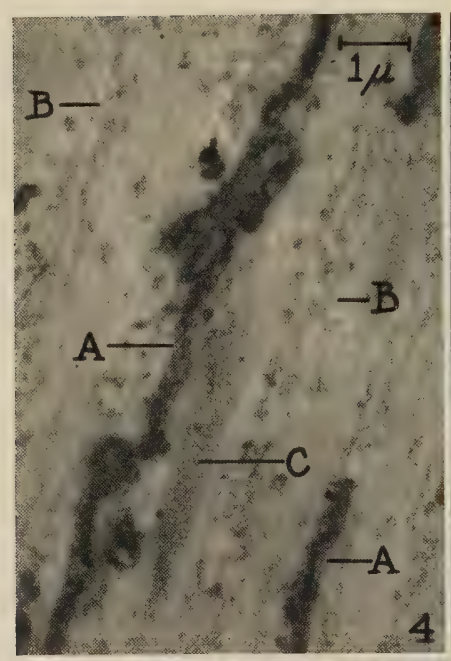
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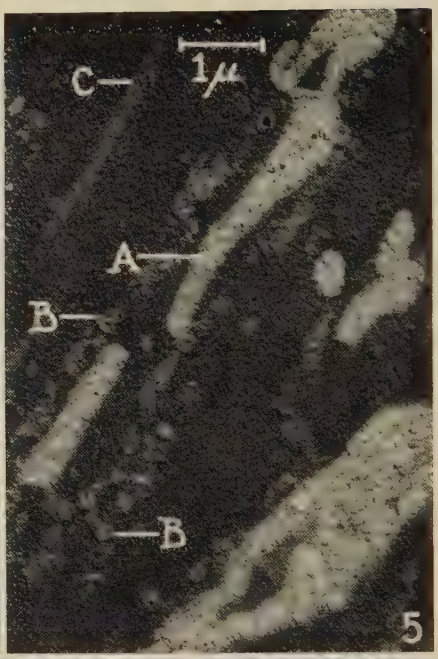
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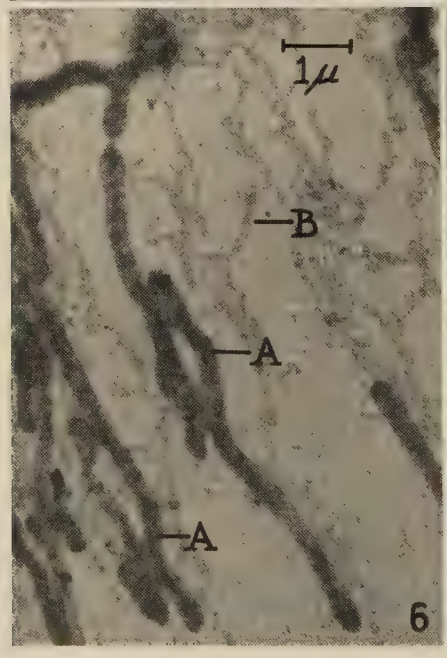
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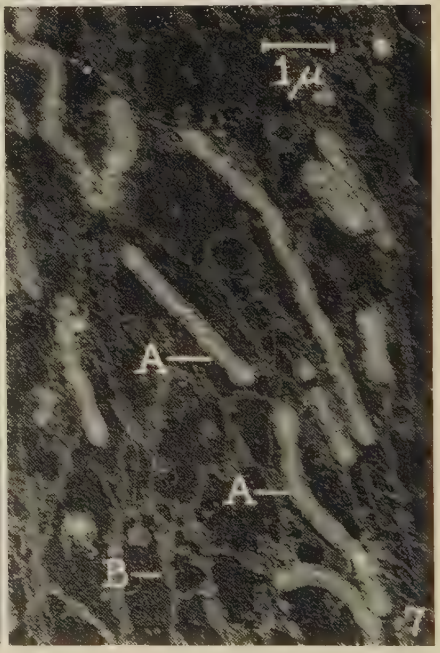
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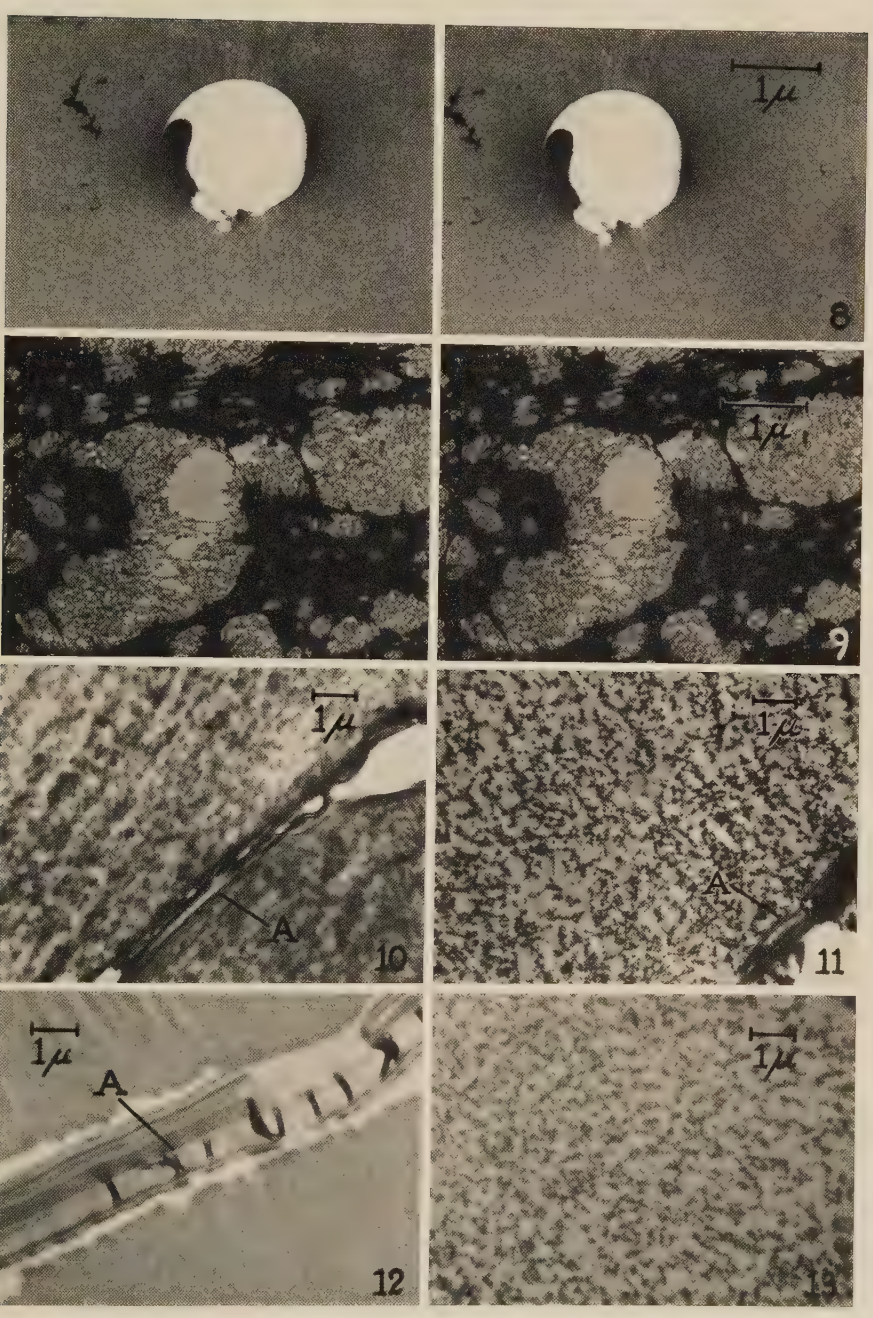
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that around the formed bodies something is present which serves to scatter electrons and blur the outlines. The limits of the mitochondria and the reticulum can be seen, but they are not sharply defined.

This observation is clarified by comparing Figs. 4 and 5 with Figs. 6 and 7. These latter micrographs show portions of identical types of cells fixed 16 hours over vapors of OsO_4. Here the mitochondria and the reticular elements are sharply defined; no fibrous condensations are evident. In the shadowed preparation (Fig. 7) the formed elements stand up as discrete bodies with shadow-casting margins. By comparison, the surface of the briefly fixed, dry cell in Fig. 5 is smooth.

It appears then, that during the period called long fixation, an amorphous matrix (and its fibrous condensations) is removed from around the formed bodies leaving them clean and available for more detailed study. This in

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Fig. 4. Electron micrograph of a portion of a cultured rabbit endothelial cell fixed for 10 minutes in vapors of OsO_4 all the while bathed in balanced salt solution. Following fixation it was washed in H_2O for 2 minutes and then transferred to the usual grids for electron microscopy. Mitochondria are indicated at *A*, strands of endoplasmic reticulum at *B*, and a small fibrous condensation of the cytoplasmic matrix at *C*. Diffuse margins of images of formed structures define the presence of electron-scattering material around these structures. Mag. 9000 ×.

Fig. 5. Portion of cell of type similar to that shown in Fig. 4 except that here the cell was shadowed with chromium, and this is the negative image. *A* marks a mitochondrion, *B* some element of the reticulum, and *C* a faint fiber array. Mag. 10500 ×.

Fig. 6. Portion of another rabbit endothelial cell fixed for 16 hours in vapors of OsO_4 while bathed in balanced salt solution. In contrast to their images in Figs. 4 and 5, the margins of mitochondria (*A*) and endoplasmic reticulum (*B*) are here sharply defined. This indicates that during the extended fixation, the electron-scattering, more or less homogeneous matrix has been removed. Mag. 9000 ×.

Fig. 7. Portion of similar cell treated the same as that in Fig. 6 except that here it was shadowed with chromium to bring out the elevation of the margins of the formed structures, which in the briefly fixed cell (Fig. 5) is concealed by the matrix. Mag. 9000 ×.

Fig. 8. Stereoscopic pair of micrographs of a film of bovine albumin fixed in vapors of OsO_4. The film was formed by flooding a 10 per cent solution of the protein over large, 1 × 2 cm pieces of celloidin-coated copper mesh and thereafter fixing for 2 hours. Following a washing in water, the films were processed by Anderson's technique for the preservation of three-dimensional structure. An apparently structureless gel is evident. Mag. 11500 ×.

Fig. 9. Stereoscopic pair of micrographs of albumin film similarly prepared except that in this case the protein was fixed in Flemming's solution. A coarse pattern of coagulation resulted. Mag. 11500 ×.

Fig. 10. Micrograph of thin section through pith impregnated with globulin and thereafter fixed in 2 per cent OsO_4 in Tyrode. There is obviously some coagulation in the gel structure but it has not resulted in the dense particle formation evident in Fig. 11 after Flemming fixation. *A* marks the wall of a pith cell. Mag. 5625 ×.

Fig. 11. Micrograph of thin section through identically prepared globulin-pith, but fixed with Flemming's solution. A dense precipitate is evident. *A* marks the wall of a pith cell. Mag. 5625 ×.

Fig. 12. Micrograph of thin section of albumin-impregnated pith fixed in 2 per cent OsO_4 in Tyrode. The gel that was formed by the fixative is seen to be remarkably homogeneous and free of evidence of coagulation. *A* marks the pith cell wall. Mag. 5625 ×.

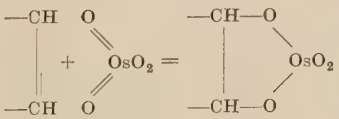
Fig. 13. Micrograph of thin section of albumin-impregnated pith fixed in Flemming's solution. Pattern of coarse coagulation is clearly evident. Mag. 5625 ×.

the vernacular of the cytologist, would be differentiation by extraction. What remains of the cell may be thought of as a membrane skeleton. Presumably the materials of the matrix are decomposed to a state which allows them to diffuse out of the cells. In support of this, it has been noted that as fixation is extended in time, the small quantity of balanced salt solution surrounding the cells acquires the brownish tint of compounds containing lower oxides of osmium. The increased fragility and relative emptiness which cultured cells show after these long fixations is probably analogous to the over-fixation effects noted by histologists (17).

The time taken to obtain this extraction effect with OsO_4 differs somewhat for different tissue. In general, cells from adult tissue explants require a longer fixation than cells from embryonic tissue. The most obvious assumption to make in this regard, is that the original hydration of the cell (higher in embryonic cells than in adult cells) determines the difference.

Chemistry of fixation. Since the cell is chemically so heterogeneous, little can be learned of the chemical nature of OsO_4 fixation from observations of its action on cells. Perhaps, therefore, the most useful alternative is to examine the action of the fixative on various relatively well-known homogeneous materials similar to or identical with cell components.

In the case of lipids, it has been assumed for many years that the reaction was a simple oxidation whereby the cell's lipids were oxidized and black osmium dioxide was deposited at the site of the reaction. Baker is more specific. He states that the OsO_4 reacts with olein which, as an unsaturated compound, reduces the tetroxide to a blackened lower oxide or hydroxide. Saturated fatty acids and OsO_4 do not react. As mentioned above, the basis for this distinction appears to reside in the particular tendency of OsO_4 to combine at ethylenic double bonds and form unstable osmium esters (8, 9).



It appears, however, that the reagent may find other chemical configurations with which to react (2). Thus the initial, rapid combination with unsaturated fatty acids may be followed by various other slower oxidation processes, as at aromatic double bonds, to give the varying degrees of osmium densities evident in both light and electron microscope images.

Less still is known of the action of OsO_4 with proteins. Hardy (14) fixed membranes of various colloidal solutions including egg white and gelatin

in: 1 per cent OsO_4 solution, OsO_4 vapors, corrosive sublimate, formalin, and dichromate, in an attempt to study fixation on physical systems. He reported that osmium vapors produced the finest fixation of any of the reagents tested. Monckeberg and Bethe (18) found that when they mixed solutions of egg white and OsO_4, no precipitation occurred, although films of egg white were fixed by osmium vapors in so far as they were no longer soluble after this action. However, no change in the films was evident microscopically.

Studies of the addition of OsO_4 to protein solution have been made by several investigators (13, 6, 3). In each instance, however, the protein preparations were far from pure, and no data were given as to concentrations or quantities of the reactant. For these reasons, it has been considered of some value to repeat and extend such observations in order to see in what respects if any, the behavior of proteins under OsO_4 fixation duplicated the behavior of cells in so far as the latter can be observed.

Solutions of 2 per cent OsO_4 and various concentrations of bovine serum albumin, bovine serum globulin, and bovine fibrinogen[1] were mixed in constant proportions of two parts protein solution to one part OsO_4. Observations were made on the rate of gelation and, where feasible, photometric determinations of opacity were recorded (see Table I). All mixtures were made in tubes of uniform size or in 1 cm square cuvettes of the Beckman spectrophotometer. It can be seen from Table I that OsO_4 brought about gelation of all three proteins when sufficient protein was present. As the concentration of protein was reduced, the gelation time was prolonged until, at the lower concentrations no gel formed. The lowest concentration of protein which produced a gel under the conditions of the experiment was different for each, as were also the speeds of the reactions and the characteristics of the gels.

Gelation proceeded most slowly in the case of albumin. From an initial viscosity of that of the protein solution, the mixture became more and more viscous until it gelled. The pH of the mixture shifted from 7.4 to 6.4 during gel formation. The OsO_4-protein mixture blackened progressively, but showed no evidence of light scattering. It was found necessary to use light of 900 mμ wave length in order to obtain a relatively high percentage transmission up to gelation time. Opaqueness such as can be developed by coagulating the protein with alcohol clearly affected the transmission of this wave length more than the darkening due to reduction of OsO_4 (Table I).

[1] Bovine plasma albumin (Armour) 97 per cent albumin. Bovine gamma globulin (Armour) Fraction II from bovine plasma. Bovine fibrinogen (Armour) Fraction I from bovine plasma. 40 to 50 per cent Na citrate ca. 70 per cent clottable N.

TABLE I

Reaction of OsO_4 and plasma proteins.

	Final protein concentration in gm per cent	Gelation time	Opacity (expressed in per cent transmission at 900 mμ)		Characteristics of gel	Subsequent behaviour
			Sol	Gel		
Albumin (in water)	20	12 min.	90	84	Clear, dark brown, firm.	Eventually liquified after several weeks.
	16	40 min.	90	74.5	Clear, dark brown, less firm.	»
	12	12 hrs.	92	(79) (not quite a gel)	Clear, dark brown, soft.	»
	8	None	93	(86)	—	
Globulin (in .85 per cent NaCl)	10	90 sec.	80	3	White, turning to brown, soft.	Gel persists
	8	120 »	Opaque		»	»
	6	Never gelled.	Developed a flocculent precipitate		White to brown.	—
	4	»	»		»	—
Fibrinogen (in Tyrode solution)	2.1	10 sec.	Opaque		White, quickly turning brown to black, soft.	Becomes fluid after several days.
	1.7	12 »	»		»	»
	1.3	20 »	»		»	»
	.85	120 »	»		Very soft.	Fluid after 24 hours.
	.43	Never gelled	—		—	—
	Control 15 gm. per cent albumin plus 50–60 per cent ethyl alcohol	Gelation with development of opacity	97 (15 mg. per cent protein alone)	3	Firm, opalescent to opaque.	

In each case 1 cc of 2 per cent OsO_4 was added to 2 cc of appropriate concentrations of protein solution contained in test tubes of uniform diameter. Gelation was determined by turning the tube sidewise to see if the mixture flowed. Opacity determinations were made with the Beckman spectrophotometer where feasible. The sol measurements were taken one minute after the osmium-protein solutions were mixed. The osmium solution was made up in .85 per cent NaCl for use with serum globulin and fibrinogen, while with serum albumin it was dissolved in water.

The reaction leading to gel formation was more rapid in the case of globulin and the product was in each case opaque. This doubtless defines some degree of coagulation.

With fibrinogen, the gelation was almost instantaneous at the higher concentrations, and as in the case of globulin, the gels were uniformly opaque. They did not appear under macroscopic examination to have the fibrous character of fibrin clots.

Although not indicated in Table I, it was also found that if the amount of protein was kept constant and the concentration of OsO_4 diminished, a lower limit was reached at or beyond which gelation would not take place. This is interpreted as indicating a possible stoichiometric relation in that part of the protein-OsO_4 reaction leading to gel formation.

The reaction of these protein solutions with OsO_4 clearly paralleled in at least two respects the apparent response or reaction of the cells to OsO_4. They initially gelled and subsequently, under the continued action of OsO_4, they (albumin and fibrinogen) decomposed and returned to the liquid state. In the case of albumin and globulin, the concentrations giving gels are not vastly different from the over-all concentration of proteins in the cell. These simularities prompted a further examination of the protein gels with the aim of further elucidating the nature and quality of fixation achieved with OsO_4.

The general appearance of the gels in itself provides some information in this regard. For example, those developed with albumin remain clear, indicating an extremely fine micellar or even unimolecular bonding. Globulin and fibrinogen on the other hand, become opaque, demonstrating a coarser unit structure.

In a further analysis of their form, 10 per cent solutions of bovine globulin and albumin were flooded over large (1×2 cm) pieces of celloidin-coated copper mesh to form protein films. Some of these were then fixed for 2 hours in vapors of OsO_4 and others, for purposes of comparison, in Flemming's solution. After washing in H_2O, they were processed for stereoscopic electron microscopy according to the method of Anderson (1). The results with albumin are shown in Figs. 8 and 9. The difference in the smoothness of the fixation of the protein with the two fixatives is striking. Three dimensional structure may be seen in both preparations, but in the case of the film fixed in Flemming's, there has resulted an extremely coarse coagulation. By contrast, the gel formed by the action of OsO_4 vapors on the albumin film is remarkably homogeneous. Similar differences were obtained with films of 10 per cent globulin, though here the OsO_4-fixed film was not so smooth and structureless as that obtained with albumin. It is readily seen that the

differences are analogous to those shown between cells fixed in OsO_4 and more destructive reagents.

With a similar purpose in mind, small, 1–2 mm cubes of pith were impregnated under vacuum with 35[1] per cent solutions of albumin and 15 per cent globulin, and thereafter fixed 4 hours in OsO_4 or in Flemming's. Following a period of washing, the blocks were taken up through alcohols and imbedded in *n*-butyl-methacrylate. Thin sections of these were cut and studied with the electron microscope. The results, as shown in Figs. 10–13, are more or less as expected. Even though subjected to alcohol and methacrylate treatments, the albumin gel is too finely constructed to show any component units at 15000 diameters. Blocks fixed in Flemming's solution but otherwise treated identically show pronounced evidence of coagulation. The globulin, as predictable from the gels formed in test tubes, is not as finely dispersed after OsO_4, but the result is somewhat better than with Flemming's solution.

From these simple experiments it is seen that OsO_4 reacts with certain plasma proteins to form gels which are remarkably free from evidence of coarse coagulation. From this and aforementioned evidence, it is probable that it has a similar effect on proteins and lipoproteins in the cell, and thus brings about their relatively faithful fixation. This process may be non-specific, such as that produced by alkalinization and acidification in experiments reported by Barbu and Machebœuf (4), but it may also be a specific action of OsO_4. Electron micrographs indicate that the effect of OsO_4 differs significantly from that of Flemming's solution, or for that matter, from most cytological fixatives.

Lack of precise knowledge of the structure of proteins, and some uncertainty as to their purity, are of course barriers to study of the chemistry of their interactions. It is worth noting, however, from Table I that fibrinogen and globulin react with OsO_4 to form gels at lower concentrations and at much more rapid rates than with albumin. An examination of the amino acid content of these proteins reveals that differences in the amount of tryptophane roughly parallel the differences in reactivity of the proteins with OsO_4 to form gels. In view of the configuration of the tryptophane molecule and the availability of double bond positions for OsO_4 interaction, it seems reasonable to assign a role in the gelation phenomenon to this particular component. It is not unlikely that the OsO_4 interacts with the complex protein molecules in a number of different ways. The progress of the reaction

[1] 7.5 per cent albumin infiltrated into pith, fixed in OsO_4, sectioned, and examined in the electron microscope, was found to be similar to 35 per cent albumin in being smoothly fixed.

in the case of albumin was reflected in viscosity changes over relatively long periods, the mixtures becoming progressively more viscous until they gelled. Even after gel formation, the reaction appears to continue, resulting finally in a reversal of the gelation. This is interpreted as indicating that the excess OsO_4 further acts on the protein to bring about a lysis of the gel micelles.

From present data, it is reasonable to propose that in osmium tetroxide fixation, there occurs an initial partial denaturation of proteins with a concurrent gelation which renders insoluble many components of the cell. This may be followed by a further denaturation and a final hydrolysis of certain proteins producing soluble end products which are capable of being washed out of the cell after long fixation. It may be supposed that the gelation phenomenon is produced by a linking together of proteins and lipoproteins by way of terminal groups and side chains capable of reacting with OsO_4. A two-step system was postulated by Berg (5), who believed that OsO_4 caused a gelation in the cell by binding together amino groups of the protein, and that with more prolonged treatment the osmium tetroxide caused the oxidation of the compound previously formed, during which time osmium tetroxide was reduced to the black, lower oxide.

The preceding discussion, if nothing else, emphasizes the lack of precise chemical knowledge concerning the nature of the combinations which form between osmium tetroxide and the proteins and other cell constituents. It is to be hoped that these reactions and others between osmium tetroxide and amino groups, nucleic acids, and lipids will come to be thoroughly investigated and better understood, for at least in electron microscopy, a great deal of the ultimate interpretation of the images of osmium-fixed material will depend on the availability of such information.

SUMMARY

Osmium tetroxide has been demonstrated to produce excellent fixation of the cytoplasm of cells, particularly for those to be examined in the electron microscope. The action of osmium tetroxide on various tissue constituents differs in its rate and nature. The reaction with lipids is probably by way of an affinity of osmium tetroxide for double bond configurations, thus forming unstable osmium esters which decompose to deposit osmium oxides or hydroxides at the site of activity.

Little is known of the reaction of osmium tetroxide with proteins, but in preliminary studies it has been shown to produce quickly an initial gelation

of protein solutions which, in the case of albumin, show no well-defined structure in the electron microscope. Coagulation images are, however, evident after other fixing agents. It is postulated that by virtue of its tetrahedral configuration, OsO_4 may form polymer-like structures with proteins establishing linkages at double bond containing groups. This initial gelation may then be followed by a further oxidation and the production of soluble end products, which after long fixation wash out of the cell.

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THE SENSITIVITY OF THE CELL TO X-RAYS

S. TIMONEN

Women's Clinic, and the Department of Anatomy, University of Helsinki, Finland

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The aim of the present study has been to elucidate the much discussed problem, which of the mitotic phases of the cell is most sensitive to ionizing radiation. To arrest the cells at the various stages, indole-3-acetic acid and colchicine have been applied respectively. In the course of the study a number of new problems emerged. These were concerned on one hand with the combined effects of the agents used, and on the other hand with the sensitivity of the different chromosome regions to x-rays.

MATERIAL AND METHODS

Commercial onion bulbs (*Allium cepa*) of Dutch origin were used. The experiments were carried out during the period January–April, 1951. Bulbs of intermediate sizes were chosen and put over glass jars containing tap water. All the experiments and irradiations were carried out at the same temperature of 20–22° C. When the roots had reached a length of 2–3 cm, the bulbs with the thickest roots were chosen for the experiments. Experience showed that the thickest roots reacted best to the treatment.

Cytological methods. As fixative acetic alcohol 1 : 3 was used, the fixation time being 1 hour. The staining and preparation was carried out with the Feulgen squash technique according to Darlington and La Cour (4).

Irradiation technique. The X-raying was carried out similarly in all the experiments: Dose 200 r, dose rate 26 r/min, 190 KW, 10 mA, filter combined 1.0 mm Cu and 1.0 mm Al, distance from the tube 60 cm, area exposed 60 cm^2. The temperature during all the experiments was 20–22° C. Roots were fixed 1, 2 and 3 days after irradiation. Shorter times of recovery were not used, since the less important physiological effects of radiation, e.g. stickiness, would have obscured the results.

X-raying during interphase. The dividing cells in the root tip meristem cease to divide and remain in the interphase when the roots are treated with indole-3-acetic acid (13, 23). This substance (IAA) is synthetic and its effects resemble those of the natural auxins. It must be stressed that the cells which have been stopped at interphase continue to divide unchanged after they have recovered from the treatment, a fact verified by the present author. No endomitosis takes place during this intermitotic interval, which is shown by the fact that all the meristematic cells

remain diploid. In order to arrest meristematic cells in interphase, the onion roots were kept for 24 hours in a 3 mg/l IAA-solution. After this period the roots were irradiated. At this time practically no dividing cells were present in the root tips. After irradiation the roots were put back into tap water.

Irradiation of the differentiated interphase nuclei. Cells in the vascular bundles and in the cortex are differentiated and do not under normal conditions divide. Some have become polyploid through endomitosis. With an IAA treatment they can, however, be induced to divide (13, 23). During this mitosis the chromosome content of the nuclei is revealed. In the experiments, untreated roots were irradiated and immediately transferred to an IAA solution (0.06–0.3 mg/l, depending on the season). After a few days the cells began to divide. The reactivity of the roots to heteroauxin varied clearly with season. Fixations were made after 2, 3 and 4 days (after 24 hours no divisions were visible). It must be pointed out here that the differentiated cells contained spontaneous chromosome changes resembling those caused by x-rays. For this reason the results of IAA treatment without X-rays irradiation must be used as a control.

Irradiation of the mitotic cell. Except for interphase it was not possible to bring all the cells in a whole tissue to the same mitotic phase or even to induce them to divide. The exact control of the phase is possible only in certain studies relating to meiosis. Therefore untreated root tips were irradiated. The cells in the root tips divide rapidly, especially in roots 2–3 cm in length. In addition, in some experiments roots were kept for 24 hrs. prior to irradiation in a 0.01 per cent colchicine solution. After this treatment no anaphases were visible and a high percentage of the meristematic cells represented a typical c-metaphase, which implies a paralyzed spindle. In addition, a proportion of the cells were in different phases of prophase. After irradiation the roots were allowed to recover in tap water, and fixations were made after 1, 2 and 3 days.

OBSERVATIONS

Criteria of interpretation. Many authors have pointed out the difficulties encountered in the interpretation of the results of x-ray experiments (3, 10, 11). The results depend on so many variables that their interpretation becomes highly ambiguous. To obtain exact results the haploid mitosis in pollen grains has especially been studied (e.g. *Tradescantia, Trillium*). Diploid somatic mitoses are, as a rule, more difficult to study owing to the greater number of chromosomes.

The somatic chromosome number of the *Allium* used in the present experiments was 16. A complete analysis carried out at metaphase would naturally be ideal (3). Such an analysis is, however, impossible to carry out on this material. The examination was therefore carried out in anaphase. Two facts could be made out exactly: 1) The number of acentric chromatids (fragments) and 2) the number of dicentric chromatids giving rise to bridges.

The first group is rather heterogenous, containing chromatid fragments,

Table I

Chromosomal aberrations after 200 r. R is calculated from the ratio of chromatid bridges and fragments per 100 anaphases.

Experiment	No. of cells with mitotic aberrations in 1 000 cells	No. of anaphases showing aberrations in 100 anaphases	No. of fragments in 100 anaphases	No. of chromatid bridges in 100 anaphases	R
1) Root tip cells in interphase	198	53	79	40	1.0
2) Colchicinized root tip cells	115	46	72	11	0.3
3) Untreated root tip cells...	98	45	54	19	0.7
4) Differentiated cells in interphase	35	12	20	—	0

chromosome fragments and minute fragments. Among these are a great number of fragments which actually result from a break plus reunion, for instance a sister reunion after a chromosome break. The percentage of fragments can thus be used with great reservation only, as it represents the sum of two contrary phenomena. Its origin is, however, a break and it varies in proportion to, if not linearly with, the original breaks caused by the irradiation.

Chromatid bridges represent a special case of the reunions which have led to the formation of a dicentric chromatid. Originally it has, however, in every case arisen through a break plus a reunion. As such it is the only reunion which can be discriminated with certainty in the somatic mitoses of *Allium* roots. The other reunions can often but not always be distinguished as abnormally long chromatids and constrictions. There is, however, no guarantee that even the majority of them have been observed. Therefore the ratio chromatid bridges/fragments has been considered as the basis of the discussion. All counts were made on 100 anaphases. At the same time, however, the number of disturbances per 1 000 dividing cells, including the metaphases was determined. This value gave a rough idea of the radiation sensitivity of the tissue in question. The results of all the observations are given in Table I. Ring chromatids are so rare that the few cases observed had no bearing on the evaluation of the recombination. Table I gives the values obtained on the day when the number of disturbances was maximal. In series 1 and 4 the fixations used were made 2 days after the irradiation, and in series 2 and 3 after 24 hours.

1) *Irradiation of the root tip cells in interphase.* The number of damaged cells per thousand cells was maximal when irradiation was carried out during the interphase. However, both the percentage of damaged anaphases and the number of free fragments in anaphase was about the same as in the following group. The large number of bridges reflects the great tendency to reunion.

2) *Irradiation of the colchicinized root tip cells.* The percentage of damaged cells is high in this group, too, although lower than in the preceding group. The number of fragments in anaphase is about the same but the low frequency of bridges lowers the value of the reunion coefficient to one third of that in the preceding group. The number of original breaks in group 1 must have been greater than in group 2, but a greater tendency to reunion and restitution has rendered a high proportion of them invisible.

3) *Irradiation of the untreated root tip cells.* The number of damaged cells is lower than in group 1. The frequency of anaphase fragments and bridges is somewhat lower also. It may be noticed that the tendency to reunion is almost the same as in group 1, thus deviating clearly from that found in the colchicine experiments. It can therefore be concluded that the increased radiation effect in the colchicine treatment cannot be explained by assuming that the chromosomes are either more breakable at metaphase or that the methaphase situation in itself diminishes the reunion tendency. Consequently it seems obvious that colchicine decreases the tendency to restitution and reunion. This results in a greater number of visible breaks. In addition to this effect of colchicine, the drug naturally increases the number of irregular divisions through paralysis of the spindle.

4) *Irradiation of differentiated cells which have ceased to divide.* The root cells above the apex turned out to be highly resistant to irradiation. Measured by any of the methods used their sensitivity was very low. The occurrence of spontaneous breaks and reunions makes the experiment uncertain but the tendency is obvious. In the control series the frequency of bridges was about the same, which makes the reunion coefficient very low, if it can be evaluated at all. Another interesting fact emerged, however, from these experiments. In some of the experiments x-rays seemed to strengthen the effect of IAA. To obtain an exact idea of this phenomenon the following experiment was carried out. First, the lowest effective IAA concentration which caused mitoses in the differentiated cells was determined. This was 0.06 mg/l. When x-rays alone were used no reaction took place. The use of IAA gave 51 mitoses per 100 fields of vision (obj. ×40). When the roots were first irradiated and then treated for two days in 0.06 mg/l solution

TABLE II

Mitotic frequency of the differentiated cells in 100 fields of vision.

Treatment	Total No. of cells in mitosis	No. of prophases	No. of metaphases	No. of anaphases
0.06 IAA mg/l	51	18	27	6
200 r+0.06 IAA mg/l	277	137	121	19

of IAA, 277 mitoses were counted in 100 fields of vision. In the latter experiment the majority of the cells were in prophase, whereas in the former experiment metaphases predominated. Fixations were made after 1–4 days; the values in Table II are from fixation made after 2 days. After 4 days the reaction was only a little stronger in the combined experiment. It may be mentioned that not all the bulbs showed this difference in reactivity, but only the thickrooted ones.

It may be mentioned that small doses of x-rays caused a slight increase in the frequency of mitoses prior to the intermitotic period in root tips of *Trillium* (3). This phenomenon has been explained by assuming that an increase in the unpolymerized nucleic acids in the nuclei stimulates mitoses when the dose is low. With the doses normally used, however, the inhibitory effect dominates. This opinion is born out by the observation that after irradiation, nucleic acid precursors increase in the cytoplasm (16). Carlson (1) has found that the frequency of dividing cells, especially of the prophase stages, increases after the intermitotic period. His explanation is that x-rays prevent late prophases from developing into metaphases. At the same time a number of prophases revert back into interphase. The corresponding observation in the present study cannot, however, be interpreted along these lines, since all the irradiated cells were in interphase, being differentiated cells which had ceased to divide. Whether the reason is an accumulation of ribonucleic acid (5), or some other reaction, for instance the liberation of so-called injury substances (cf. 8), is not clear for the present.

DISCUSSION

It can hardly be definitely decided in which division stage the cell is most susceptible to irradiation. Obviously meiotic chromosomes differ greatly physiologically from the mitotic chromosomes. The different reproductive and physio-

logical phenomena may take place in different phases and at different rates in various cases, and this naturally influences the sensitivity of the chromosomes to irradiation. X-ray experiments have mostly been carried out on the pollen grain mitosis of *Tradescantia*. Late interphase and early prophase have been found to be the most sensitive stages (cf. 10, 15). Discrepant results, however, have been obtained by Sparrow (19, 20, 21) who studied x-ray breakage during microsporogenesis in *Trillium*. In his experiments the first meiotic metaphases turned out to be the most sensitive stage. In the present study irradiation has damaged the root tip cells of *Allium* most severely in the interphase.

Marquardt (14) maintains that the greater sensitivity of the interphase to x-rays depends on the fact that the chromosomes are relatively poor in desoxyribonucleic acid (DNA) and "maximal aufgelockert" at this stage. Koller (10), Darlington and La Cour (3), as well as Sparrow (19, 20, 21), have also considered their results in the light of the nucleic acid cycle of the cell, and explained the variation of the breakage sensitivity as depending on this cycle. Their opinion seems well founded. Nucleic acids have been found to be of great importance for the reproduction and function of the cell. Another line of evidence is that x-rays have been shown to break the long thymonucleic acid-histone molecules (22). The simple hypothesis, however, that the frequency of breakages depends directly on the concentration of DNA, being either negatively or positively correlated with it, is by no means proved (6, 7, 12).

It is not impossible that the extended condition of the chromosomes during interphase (17) in itself causes the higher reactivity, including the x-ray sensitivity, independent of the actual amount of any compounds present, whether nucleic acids or proteins.

The greater x-ray sensitivity may be connected with the reproductive phase of the chromosomes also. If this is the case, the great difference in the x-ray sensitivity of meristematic and differentiated cells in the interphase is easy to understand. The interphase in meristematic tissues is a brief passing period of intensive chromosome reproduction while in differentiated cells it is final and all processes obviously occur at a slower rate (cf. 10, 18).

In the present work special attention has been paid to the similarity of the structural changes that have arisen spontaneously in the differentiated cells of the *Allium* root and those obtained by irradiation. The spontaneous occurrence of fragments alone would be of less importance, as their origin could be interpreted as due to degeneration. The strong tendency to reunion, however, requires some other explanation. The breaks in this case cannot

be the result of general decomposition, but must be locally restricted to certain points. This is shown by the fact that the broken ends are able to function, i.e. become restituted and reunited. An interesting explanation has been advanced by Therman (23). This is based on the idea of Huskins (9) that the reproduction of the genes is intimately connected with their function. It could be thought that in differentiated cells certain genes are inactive, and therefore do not reproduce during endomitosis. When such a cell is induced to divide the results of breakage and reunion become visible, i.e. fragments, chromatid bridges, rings and other changes are observed.

Marquardt (14) comes to the conclusion that the protein framework of the chromosomes contains "Lockerstellen" in which the end groups of the big molecules react with special ease. At these points the spontaneous and x-ray induced breaks as well as other connected phenomena would take place more easily than elsewhere.

If we combine Marquardt's ideas with the present results we arrive at the following working hypothesis.

The segment of the chromosome situated between two "Lockerstellen" is a structural unit. In reproduction it also acts as a unit. Genetically it consists of one or several genes. It is probable that the reproductive power inherent in the unit segment depends on specific chemical properties. When a chromosome is irradiated we have the following possibilities.

Irradiation destroys the reproductive power or a part of it, due to a chemical change. If the chromosome is examined immediately no visible changes are seen. In the following cell generations the change becomes manifest, as the reproductive power of the segment is destroyed. The segments adjacent to the lacking one restitute or reunite in various ways, or fragments are formed. If we assume that the chromosome is lamellar (2) in structure and only a part of it is damaged 1/2 or 1/4 or even smaller breaks in the chromatid may arise which are manifested in later cell generations.

The segment between two "Lockerstellen" is, according to this line of thought, comparatively refractory to the effects of various agents. If the irradiation is applied in the reproductive phase the injuries are probably greater than otherwise. A disturbance in the organisation of nucleoproteins might lead to stickiness or irregularity in the coiling of chromosomes. The degree of injury probably depends on the relation between the rate of irradiation and the rate of reproduction.

Direct energy absorption and indirect chemical effect on the "Lockerstellen" quickly lead to breaks according to Marquardt. The majority of breaks visible in the subsequent mitoses have arisen in this way. The substances

which are produced elsewhere in the cell due to the irradiation probably also act on the "Lockerstellen". Reunion also occurs at the broken "Lockerstellen", and is similarly determined by the physiological milieu.

The hypothetical views presented above imply that the ionizing radiation, which is assumed to be equally distributed throughout the chromosome, effects different changes in different segments of the chromosome, depending on the character of the latter. The changes caused by the smallest energy absorption which may lead to a manifest break can be classified into two main groups: loss of the reproductive power in the unit segment, or a break in the "Lockerstellen". Neither of these groups is homogeneous since the lamellar structure of the unit segments enables the injuries to take place by degrees. The mechanism of the breakage is, however, different in the two groups. Breaks in the unit segment are revealed only after reproduction of the chromosome, whereas the "Lockerstellen" may be broken either by a direct or by an indirect ionization.

SUMMARY

1) The effect of x-rays on plant cells has been studied in combination with indole-3-acetic acid and colchicine treatments. As material root tip cells of *Allium cepa* have been used.

2) The greatest sensitivity to breakage and reunion was shown by meristematic cells in the root tip which had been arrested in interphase by indole-3-acetic acid.

3) Cells which had been treated with colchicine revealed almost as many injuries as those irradiated during interphase. This is interpreted as depending on a lowered tendency to reunion due to the colchicine treatment.

4) Differentiated cells, which had ceased to divide, were induced to undergo mitosis after irradiation by a treatment with indole-3-acetic acid. The chromosomes in these cells proved very refractory to x-rays.

5) X-raying seems to strengthen the effect of indole-3-acetic acid in inducing cell division. The phenomenon became apparent with the lowest possible concentrations of indole-3-acetic acid inducing division.

6) A hypothesis is advanced concerning the mechanism of the structural changes in the chromosomes induced by x-rays. It is based on the assumption that a chromosome consists of segments which act as units in reproduction. X-rays may either break the chromosome between these segmental units or destroy the reproductive power of a segment which leads to breakage in subsequent cell divisions.

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THE DETERMINATION OF CELL VOLUME

H. LOUISE MICKLEWRIGHT[1], N. B. KURNICK[2], and R. HODES[3]

Department of Psychiatry and Neurology and Laboratory for Cell Research, Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana

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The measurement of the volume of individual cells is of importance in a variety of investigations. Some studies have been concerned with the correlation of nerve cell volume with anatomical localization (1, 2, 3, 4, 9). Others have required the measurement of volume for the estimation of chemical constituents from photometric (histochemical) data (8) and the correlation of cell growth with histochemical changes (7). Neuronal volume has been correlated with the rate of impulse conduction (11) and with susceptibility to poliomyelitis virus (6).

Determinations of cell volume cited above are based on the assumption that the cell is a spheroid (ellipsoid of rotation). Bok (1, 2) considered the cell a prolate spheroid and calculated the volume from measurements of the diameters of a section through the nuclear center ("Kernmitte"). Hodes *et al.* (6) measured cross-sectional diameters and calculated volumes from the formula for an oblate spheroid. Rather (10) determined nuclear volume from the distribution of nuclear diameters in thin section and calculated the cytoplasmic volume from the nuclear-cytoplasmic ratio according to Chalkley (5).

Since the determinations of cell volume derived by the application of formulae for oblate or prolate spheroids rest on assumptions which have not been adequately examined, it was decided to test the formulae of Bok (1) and Hodes *et al.* (6) to determine whether they were satisfactory. It will be shown that estimates of cell volume based on either of the above calculations are inaccurate. More adequate methods were therefore devised, and will be described below.

Rather's (10) method will not be considered here since we are concerned with measurements on individual cells, rather than with statistical methods.

EXPERIMENTAL PROCEDURE

A direct method for the measurement of cell volume is to reconstruct the cell in a three-dimensional figure. Five anterior horn cells were measured

[1] Aided by a grant from the National Institutes of Health, U.S. Public Health Service.
[2] Aided by grants from the American Heart Association and the National Heart Institute, National Institutes of Health, U.S. Public Health Service.
[3] Present address: Department of Zoology, University of Arizona, Tucson, Arizona.

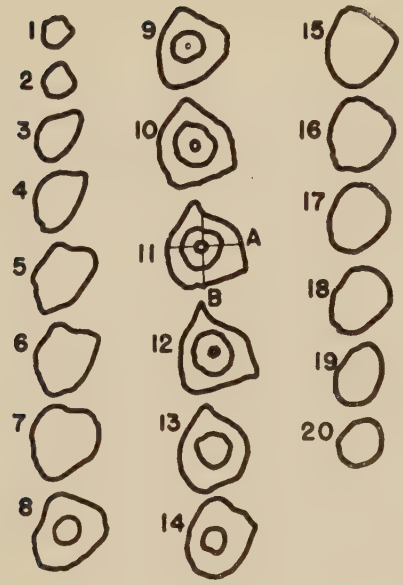

Fig. 1. Camera lucida drawings of serial optical sections of neuron from normal rhesus monkey cord (C 7). × 1600.

in 15 μ sections of normal rhesus monkey spinal cord (C 7).[1] Since the cells exceed 15 μ in thickness, it was necessary to follow them through several sections. This laborious and difficult procedure was simplified by studying thick sections. The cervical enlargement of cat spinal cord, fixed in 10 per cent formalin and embedded in paraffin, was sectioned at 60–100 μ and stained with gallocyanin.

With a 1.8 mm oil immersion objective and 10× ocular (mechanical tube length 160 mm) and camera lucida attachment, optical sections were drawn from the top to the bottom of motor horn cells at 2.5 μ steps as determined by the fine adjustment drum of the microscope. In Fig. 1 such a series of camera lucida drawings is shown. The magnification of the drawings is 1600×. The area of each optical section was measured with a Keuffel and Esser planimeter. Since each area may be considered to be the cross-sectional area of a cylinder 2.5 μ high (1.25 μ on either side of the optical section), the volume of this cylinder is the area multiplied by 2.5. The sum of these volumes, then, is the volume of the cell. Duplicate determinations of cell volume performed on six cells by repeating the entire procedure (including drawing new optical sections) agreed within 5.8 ±2.6 per cent.

For the calculation of cell volume by other methods to be discussed below, the cell thickness and the dimensions of the major and minor axes of the idealized ellipse approximated by the central optical section were measured from the camera lucida drawings. The axes selected bisected each other as appropriate for an ellipse

[1] Kindly supplied by Dr. David Bodian, Johns Hopkins University, Baltimore, Maryland.

TABLE I

Comparison of methods of measuring cell volume (21 cells) using results obtained by reconstruction method as standard.

(1) Method	(2) Range of calculated volumes $\times 10^2\ \mu^3$	(3) Range of error in per cent	(4) Mean error in per cent	(5) σ_E per cent	(6) Probable range of V*
Reconstructed Volume	54–467				
$\pi/6\ a\,b^2$	33–349	−62 to + 18	−24.2± 5.4	24.1	0.8 *D* to 3.6 *D*** mean = 1.3 *D*
$\pi/6\ a^2\,b$	68–608	−36 to +235	+23.4±12.4	55.6	0.4 *D* to 9.1 *D*, mean = 0.8 D
$\pi/6\ a\,b\,c$	56–466	−37 to + 14	− 7.6± 3.0	13.5	0.8 *D* to 1.5 *D*, mean = 1.1 D
2/3 area × *c*.....	53–400	−22 to + 19	− 1.0± 2.7	12.1	0.8 *D* to 1.3 *D*, mean = 1.0 *D*

* Range of 96 per cent of Cases ($2\,\sigma_E$) V is the reconstructed volume.

** *D* = volume calculated by various formulae.

(Section 11 — Fig. 1). The major and minor axes and the cell thickness could also be measured satisfactorily directly in the microscopic image by means of an ocular micrometer and the calibrated drum of the fine adjustment screw.

In order to visualize the shape of sections through the cell at right angles to the actual plane of section, reconstructions were drawn (Fig. 2) parallel to the major and minor axes of the central optical section (Section 11 — Fig. 1).

RESULTS AND DISCUSSION

The results of the determination of cell volume by the reconstruction method, Bok's and Hodes's formulae (using the axes of the central section as measured in the camera lucida drawing) are presented in Table I. It is noted that the formula for a prolate spheroid produces errors in the range of −62 to +18 per cent with a mean error of −24.2 ±5.4 per cent, while that for an oblate spheroid yields errors of −36 to +23.5 per cent with a mean error of +23.4 ±12.4 per cent.

Statistical tests indicate that Bok's formula gives significantly low values (X^2 test for the sign of error). It is therefore possible to improve the mean value by the application of a correction factor, although no reduction in variability can be accomplished. Since the individual errors (Table I, column 3) are calculated from the formula

$$\frac{D - V}{V} = E$$

where V is the volume determined by reconstruction, D is the volume determined by the formula in question, and E is the error of D (compared to the reconstruction method), then $V = \frac{D}{E + I}$. For Bok's formula, mean $V = 1.32\,D$.

Since the X^2 test indicated that the formula for the oblate spheroid does not significantly favor values in either direction, no correction factor could be expected to be of value.

Since neither of the longitudinal sections constructed in Fig. 2 is a circle, it follows that motor horn cells are neither prolate nor oblate spheroids oriented with both principal axes in the plane of the microtome section, as would be required for the

Fig. 2 A.

Fig. 2 B.

Fig. 2 A. Reconstruction of cell in Fig. 1 along axis "A" of Section 11, Fig. 1.
Fig. 2 B. Reconstruction of cell in Fig. 1 along axis "B" of Section 11, Fig. 1.

application of Bok's or Hodes's formulae. In general, the cells appear to be non-rotational ellipsoids. The formula for this figure is $\pi/6\,a\,b\,c$ (formula 1) where a, b, and c represent respectively the major, minor, and mean axes of the ellipsoid. The formula may also be expressed in the form 2/3 area $\times\, c$ (formula 2) where the area is that of the central section and c is the thickness of the cell.

The required dimensions were obtained from the camera lucida drawings. The range of errors for formula 1 is +14 to —37 per cent with a mean error of —7.6 ±3.0 per cent and for formula 2 the range is +19 to —22 per cent with a mean error of —1.0 ±2.7 per cent (Table I). Thus both of these formulae provide relatively narrow ranges of error with no singificant mean error and with limited variability for individual measurements (σ_E in Table I).

In view of the good correspondence of cell volumes obtained by applying formulae 1 and 2 and those determined by camera lucida reconstruction and planimetry, it was decided to apply these formulae as the standard method for the determination of nuclear volume. Although optical sectioning and planimetry are satisfactory when applied to the large, somewhat irregular cell, the smaller size and regular outline of the nucleus make the latter less amenable to this method. The tendency to draw the outlines of the top and bottom 2.5 μ sections too large (since in optical sectioning the larger areas blend into the smaller ones), while not significant for the large cell, introduces considerable errors in the small nucleus. It is not surprising, therefore, that the nuclear volumes calculated by formulae 1 and 2 are in good agreement, while those derived from the reconstruction method tend to be higher

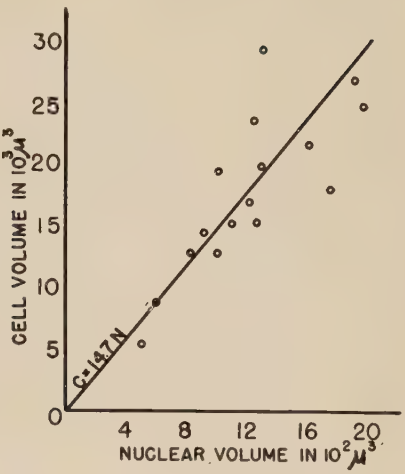

FIG 3

Fig. 3. Cell volume calculated by planimetry plotted against nuclear volume calculated by $\pi/6\ a\ b\ c$ for 16 cat motor neurons. (Mean cell volume = 14.7 × nuclear volume.)

TABLE II

Comparison of methods of calculating nuclear volume (21 cells) using $\pi/6\ a\ b\ c$ as standard.

(1) Method	(2) Range of calculated volumes $\times 10^2\ \mu^3$	(3) Range of error in per cent	(4) Mean error in per cent	(5) σ_E per cent	(6) Probable range of V*
$\pi/6\ a\ b\ c$	4.8–39				
$\pi/6\ a\ b^2$	4.2–38	−34 to +25	-12.4 ± 3.7	16.7	0.8 D** to 1.9 D, mean = 1.1 D
$\pi/6\ a^2\ b$	5.1–39	−31 to +56	-7.3 ± 4.7	21.0	0.7 D to 1.5 D, mean = 0.9 D
2/3 area × c	4.7–39	−12 to +12	-1.5 ± 1.4	6.1	0.9 D to 1.2 D, mean = 1.0 D
Reconstructed Volume	6.5–43	−9 to +46	$+14.6 \pm 3.5$	15.8	0.7 D to 1.2 D, mean = 0.9 D

* $V = \pi/6\ a\ b\ c$.
** D = volume calculated by various formulae.

(Table II). The tendency of the prolate spheroid formula is to give low values for the volume. The oblate spheroid formula tends to yield random errors with no significant mean error (Table II).

Fig. 3 depicts the relation between nuclear and total cell volume for each cell. These volumes were calculated by the most accurate methods available: the reconstruction method for the total cell volume and formula 1 for the

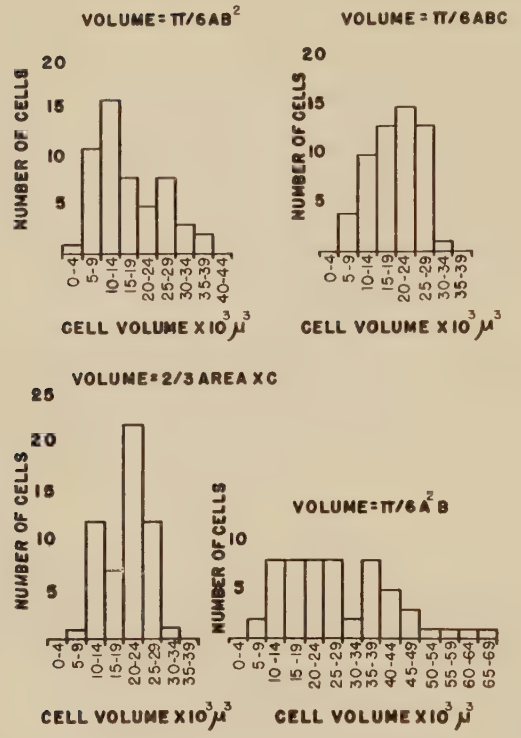

Fig. 4. Distribution of cell volume of 55 cat motor neurons determined by various methods. Formula 1 is $\pi/6\ a\,b\,c$; formula 2 is 2/3 area × c.

nuclear volume. A linear relationship appears to exist. The mean ratio of cell : nuclear volume is 14.7 ± 1.3 for cat cells. This is not in agreement with the relationship determined by Bok for cells of the cerebral cortex; namely, that cell volume is a function of the square of the nuclear volume.

Since the formulae of Bok and Hodes give fair approximations of the nuclear volume (Table II) and since there appears to be a linear relationship between cell and nuclear volume (Fig. 3), it might be expected that the measurement of nuclear volume by either of the above formulae could be used for the calculation of cell volume. These methods require the measurement of only two dimensions and would permit the use of thin sections and the recalculation of previously accumulated data in the literature. However, this procedure proves to have no advantage over the application of the original formulae to the estimation of cell volume because of the considerable variability of individual cell : nuclear volume ratios (Fig. 3), and the errors in the determination of nuclear volumes.

In some investigations, the individual cell sizes may not be of great im-

portance, but the distribution of cell volumes in a particular population may be required. The values of thirty-nine additional cat motor horn cells were therefore measured in order to provide a sufficiently large sample for evaluating the distribution of cell sizes in the cat's motor horn. The reconstruction method was not employed for these additional cells. In Fig. 4, we have graphically presented the distribution of cell volumes of all 55 cat motor neurones as determined by the several methods we have investigated (reconstruction method omitted because of small number of cells measured). It is apparent that formulae 1 and 2 yield similar curves, each representing a single "normal" population with a peak at 20000–24000 μ^3. The spheroid formulae give rather different volume distributions for the same 55 cells. Futhermore, in the case of the formula $\pi/6\ a^2\ b$, it is no longer apparent whether one or two populations (or types) best fit the data.

CONCLUSIONS AND SUMMARY

The investigation of previously applied formulae for the measurement of nerve cell volume demonstrates their unreliability as applied to motor horn cells of the cat. The formula for a prolate spheroid (1) produces errors such that the correct volume may fall in the range of 0.8 to 3.6 times the calculated volume. The formula for an oblate spheroid (6) produces errors which cause the correct volume to fall in the range of 0.4 to 9.1 times the calculated value.

The reconstruction method described in this paper is the most accurate method for the determination of cell volume. Except for special purposes, however, it is too laborious to be practicable. A good approximation of the cell volume may be calculated by either of two formulae (2/3 area $\times$ c or $\pi/6\ a\ b\ c$) from the following measurements on thick (60–100 μ) sections: the thickness of the cell and either the area or the axes of the central cross-section of the cell. Sections of 60–100 μ thickness are chosen so as to minimize the preferential selection of small cells for measurement. By the use of the two formulae suggested in this paper (see p. 153, 154) it is found that the correct volume rarely falls outside the range 0.8 to 1.5 times the calculated value. Ease of application favors formula 1, although formula 2 appears slightly more accurate.

A linear relationship is found between nuclear volume and cell volume ($14.7 \pm 1.3 \times$ nuclear volume).

By the methods proposed by us, the distribution of volumes of 55 anterior horn cells of the cat indicates a normal population curve. This curve is

distorted as a result of the erroneous assumptions of earlier formulae when these are applied.

The assumption that the anterior horn cell approximates a non-rotational ellipsoid with three independent axes appears to fit the data obtained from reconstructions. Furthermore, the assumption often made (1, 2, 6) that sections through the nucleolus must represent the central plane is not borne out by our observations. The nucleolus is frequently considerably eccentric in relation to both nucleus and whole cell.

The calculation of cell volume by the two formulae presented is probably applicable to cells of other tissues.

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SPECIFIC STAINS FOR ELECTRON MICROSCOPY

W. G. P. LAMB, J. STUART-WEBB, L. G. BELL, R. BOVEY, and J. F. DANIELLI

Departments of Physics and Zoology, King's College, London, England

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The increasing use of electron microscopy in cytological work has indicated the need for developing special techniques for staining particular regions of specimens. The use of higher accelerating voltages of the order of from 100 KV to 400 KV promises to render the majority of specimens more transparent than has hitherto been attainable, and has encouraged the view that methods or stains, as they have been termed, which increase the opacity of regions or components of specimens would facilitate the elucidation of morphological detail and enable measurements to be made on the concentrations of those chemical groups concerned in the reaction.

Colour stains have been used in light microscopy for many years and lately a few attempts have been made to adapt some of these techniques to produce the required increase of opacity.

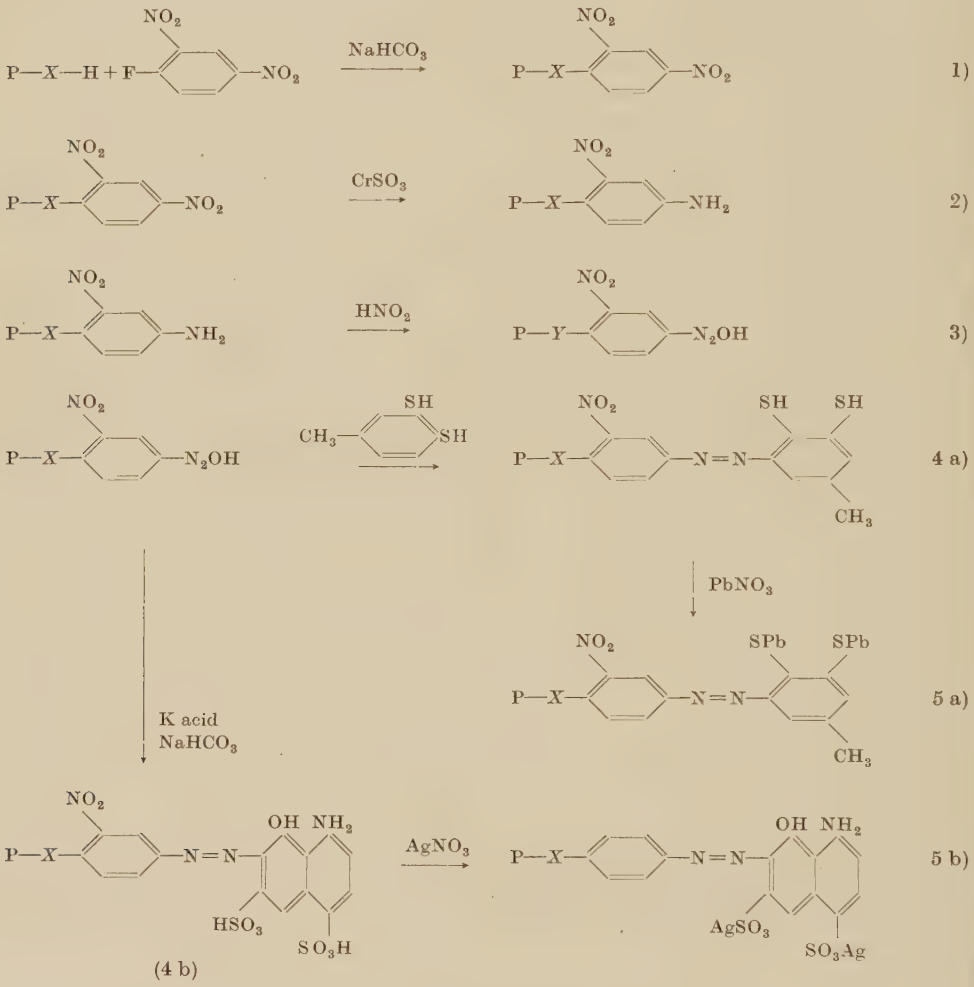
Schmitt (7) has used phosphotungstic acid to stain bands of material in collagen fibres, while Calvet, Siegel and Stern (1) have used lanthanum acetate to stain debris of nuclear material from thymocytes which had been exploded in a special vacuum device. Claude and Porter (5) have used osmium tetroxide as a combined fixative and stain in their work on the electron microscopy of tissue culture cells. These methods suffered from the disadvantage that they were non-specific in action.

Recently, Danielli and Bell (2, 3, 4) have developed methods of staining specific groups of protein molecules for light microscopy. In this paper a description is given of an attempt which has been made to extend these methods for electron microscopy.

MATERIAL AND METHODS

Chemical Procedure. As outlined by Danielli (loc. cit.), the method consists in treating the specimen with a reagent which will react with specific groups in the protein side-chains and to which may be added other groups to increase the electron scattering power of the molecules.

The reagent used was 1-fluoro-2 : 4-Dinitrobenzene referred to as 'DNFB', which Sanger (6) has shown will react with NH_2 groups, SH groups, the phenolic group of tyrosine and also more slowly with histidine. 'P' being used to signify a protein molecule and '*XH*' a reactive group, the following equations illustrate the chemical treatment:



The initial condensation product of 1) is reduced with chromous sulphate 2), diazotised with nitrous acid 3) and then coupled with either (a) 4-methyl-1 : 2-mercaptobenzene 4) which is treated with lead nitrate to give the lead salt 5), or (b) with K acid (1-amino-8-hydroxynaphthalene-4 : 6-disulphonic acid) which is treated with silver nitrate to give the silver salt.

Blank or control experiments were carried out by treating some of the specimens with the metal solutions at the end of stage 1). This was done to eliminate any possible reaction between the metal salts and the original reactive groups in the protein molecule.

Biological Material. Experiments were first made with thin gelatine films supported by formvar films on glass slides. Difficulty was experienced in preparing films which were sufficiently uniform in thickness, and it was found that the films did not survive the chemical treatment as partial destruction was caused. Following a suggestion by Prof. J. T. Randall, bull spermatozoa were found to be suitable, since they survived the treatment and measurements taken on a large number of untreated spermatozoa had shown that they were fairly uniform in opacity.

At first, the sperm were treated after they had been mounted on glass slides covered with formvar films, these being dipped into the reagent solution. It was found, however, that the alcohol used in the DNFB solution attacked the films and invalidated the results. Subsequently, the material was treated in bulk, being separated from the reagents by centrifugation, and then mounted on a uniform formvar film for examination.

Physical Measurements. The opacity determination of the material at the various stages of the treatment involved its examination in the electron microscope under known and reproducible conditions. These included the operation of the microscope at a fixed acceleration voltage (100 KV) and with a certain angular aperture of the illuminating beam. The object was inserted at a reproducible distance from the objective lens and the same objective aperture used throughout.

The specimens were photographed on a high contrast plate which was developed for a fixed time in Ilford ID 2 developer of known strength. The photographic densities of the objects and immediate backgrounds were measured with an extinction photometer and their opacities computed by reference to the characteristic curves of the photographic plates.

RESULTS

Both the K acid and dithiol reactions were observed to give coloured stains under the light microscope. The greatest degree of stain appeared to be in the mid-piece and for this reason most of the opacity measurements were made on that region.

Table I shows figures for the opacity of the mid-pieces of bull sperm after each stage of the treatment, together with the number of individual determinations and the standard deviation (7) of an individual from the mean. The untreated material had a mean opacity of 0.825, while the completely treated material gave figures of 1.21 and 1.20 respectively, after all the groups including either the silver or lead atoms had been added. The corresponding figure for those controls which had been treated only with DNFB followed by the metal salt was 0.88 in each case. The values for the opacities of the stained material represent a significant increase over those for the untreated.

TABLE I

Treatment	Opacity (mean value)	σ	Number of individual determinations
None	0.825	0.07	24
DNFB	0.950	0.109	18
$CrSO_3$	1.075	0.064	22
HNO_2	0.873	0.068	23
K acid	0.945	0.062	22
$AgNO_3$	1.210	0.132	20
Dithiol	1.000	0.072	22
$PbNO_3$	1.200	0.134	21
DNFB + $AgNO_3$	0.880	0.071	18
DNFB + $PbNO_3$	0.880	0.041	20

An attempt has been made to deduce the proportion of reactive material in the specimen from the measured increase in opacity, the atomic numbers of the atoms or groups which have been added being known. If the scattering power of individual atoms is taken to be proportional to Z_3^1, where Z is the atomic number, it is well nigh impossible to calculate the relative number of reactive groups, unless the composition is known. However, if the scattering power is taken to be dependent on the first power of Z, this ratio may be computed.

Consider first the case of the K acid and silver stain: let x be the total atomic number of the protein molecule and other unreactive material in the specimen per group which reacts with one staining group. If it is assumed that the opacity is proportional to the combined atomic number, the ratio of opacities before and after treatment may be equated to the ratio of the atomic numbers. Since the total atomic number of the staining group $C_6H_3(NO_2)\,N_2C_{10}H_4\,(OH)(NH_2)(SO_3Ag)_2$ is 332

$$\frac{x + 332}{x} = \frac{1.21}{0.88}$$

$$\text{whence } x = 885.$$

Secondly, for the dithiol and lead stain, the total atomic number of the group $-C_6H_3(NO_2)\cdot N_2\cdot C_6H_2(CH_3)(SPb)_2$ is 319, so that in this case:

$$\frac{x_1 + 319}{x_1} = \frac{1.20}{0.88}$$

$$\text{whence } x_1 = 887.$$

Free α-amino groups being neglected for the moment, the atomic numbers of the reactive amino-acids residues present in the material lie between 86 for tyrosine and 54 for cysteine, the others — arginine, lysine and histidine — having atomic numbers of 76, 70 and 72 respectively. The proportion of reactive residue in the material will thus lie between $86/880 = 10$ per cent (if the material were composed solely of tyrosine) and $54/880 = 6$ per cent (if the material were composed solely of cysteine), the average value of 880 being taken for x. The presence of free α-amino groups will not affect these figures provided that they are distributed through residues of different types and not confined to those outside the above range (e.g. glycine, $Z = 30$).

That the proportion of reactive groups to the total amount of material in the mid-piece lies between 10 and 6 per cent is based on the assumption that the opacity is proportional to the first power of the atomic number Z; in fact, the scattering is more nearly proportional to $Z^{\frac{1}{3}}$ so that the above figures are somewhat high.

SUMMARY

A specific method of staining certain groups in proteins for examination in the light microscope has been developed for electron microscopy.

The compound DNFB (1-fluoro-2:4-dinitrobenzene) reacts with NH_2, SH and phenolic groups, and may be made in turn to react with further groups and the heavy metal atoms Pb and Ag. These metals, together with the intermediate groups added, were found to provide an increased opacity in the material. Measurements were made on the mid-piece of bull spermatozoa, which proved to be convenient material for the work.

An approximate calculation indicates that between 6 and 10 per cent of the material in the middle piece is able to react with DNFB.

We are indebted to Prof. J. T. Randall for most helpful advice and criticism and to Dr. J. Walton for his very kind assistance in obtaining supplies of bull spermatozoa. The work was made possible by grants from the British Empire Cancer Campaign to three of us (L.G.B., W.G.P.L., J.S–W.).

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AUTORADIOGRAPHIC DETECTION OF INCORPORATION OF P^{32} INTO CHROMOSOMES DURING MEIOSIS AND MITOSIS

J. H. TAYLOR

Biology Division, Oak Ridge National Laboratory[1], *Oak Ridge, Tennessee*

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Autoradiographs that detect radioisotopes in individual nuclei or even single chromosomes offer unusual opportunities to study the time and rate of incorporation of certain elements into cellular constituents. A technique that gives a resolution of this magnitude has been described recently (5). Its application to cells of bean roots has revealed uptake of P^{32} and S^{35} in a fraction of the chromosome not extracted by fixation in alcohol-acetic acid and hydrolysis with *N* HCl (6, 7). Since desoxyribonuclease removed the phosphorus from this fraction, the unextracted P^{32} is believed to be bound in a desoxypentose nucleic acid. Most of the S^{35} is presumed to be bound in protein. These two isotopes are not incorporated during cell division, but during some part of interphase. Since the incorporation is not continuous, uptake is thought to represent periods of synthesis of chromosomal material. Thus, a new method for detecting the time of duplication of the chromosomes appears to be available. Further testing of this technique on sporogenous cells of anthers seemed advisable. The synchronized development during meiosis, the subsequent long interphase, and the mitosis in the microspore provide material that may be exposed to isotopes at any desired stage in the division cycle. Incorporation of P^{32} may be studied during both meiotic and mitotic division cycles.

MATERIALS AND METHODS

Buds of *Tradescantia paludosa* (Sax's Clone 5) and *Lilium longiflorum* var. "Floridii" were used as a source of cells in stages of meiosis and mitosis. Ends of freshly cut

[1] This work was done while the author was a member of the Department of Botany, University of Tennessee and a consultant at Oak Ridge National Laboratory under Contract No. W-7405-eng-26 from the Atomic Energy Commission. Present address is Department of Botany, Columbia University.

Appreciation is expressed to Dr. G. R. Noggle for assistance in handling the P^{32} and in the preparation of the manuscript.

inflorescences of these plants were immersed in solutions of phosphate ($Na_2HPO_4 \cdot 7H_2O$) containing P^{32} at activities of 20–75 μc/ml. For most experiments the phosphate solution had an activity of 20 μc/ml and contained 0.1 mg of the salt/ml. The P^{32} reaches the buds in 2–3 hours. At intervals of 8–48 hours after the P^{32} reached the buds, cells from the anthers were smeared on microscope slides, fixed in alcohol-acetic acid (3:1) for 24 hours, passed through 70, 50, and 30 per cent ethyl alcohol, hydrolyzed in *N* HCl for 10 minutes at 60° C, stained with the Feulgen reagent and after 1 hour or more of washing in several changes of distilled water, coated with a stripped emulsion layer (5) (Kodak, Ltd., Kingsway, London, England) in the darkroom and exposed at 4° C for 14 days in bakelite slide boxes containing "Drierite" and sealed with Scotch tape. These slides were developed in D-19 for 5 minutes at 18° C, washed and fixed for 10 minutes in Kodak acid fixer at the same temperature. The slides were then washed for 30 minutes in running water at 20° C or below, dehydrated in an alcohol series, cleared in xylene and mounted in balsam or "Clarite-X" dissolved in xylene. The Feulgen stain withstands the developer and fixer.

Perchloric acid (10) may be used for differential extraction of the phosphorus compounds from cells. Four anthers from one bud were smeared on different slides. One was fixed and handled as described above and the other 3 were fixed in cold 70 per cent alcohol for 1 hour and then passed through the following solutions: 5 minutes in cold 70 per cent alcohol with $HClO_4$ added to make a 0.1 *N* solution alcohol-ether (3:1) for 6 minutes at 70° C; and cold 0.2 *N* $HClO_4$ for 5 minutes. One slide was removed to distilled water at this point. Cells on this slide retain both pentosenucleic acid (PNA) and desoxypentosenucleic acid (DNA) phosphorus. The remaining two were left 12 to 16 hours in cold 1 *N* $HClO_4$. This removes the PNA phosphorus. One was transferred to distilled water and the other was placed in 0.5 *N* $HClO_4$ for 20 minutes at 70° C. This last slide from which all PNA and DNA phosphorus had been removed was then rinsed in distilled water. Autoradiographs were prepared from each slide as described in the first schedule. These slides were examined with the phase microscope because the cells were not stained.

RESULTS

1. *General observations.* P^{32} is detected by the presence of developed grains in the emulsion above cells containing this isotope. Ten or more grains produced above the nucleus can be detected as a positive autoradiograph (5) by inspection. The striking absence of activity in most nuclei and the high activity in certain others made counting of grains unnecessary (Figs. 1, 2). Autoradiographs could be recorded as positive or negative. The period of incorporation is usually relatively short because exposures that varied only in time (8–48 hours) produced autoradiographs of nearly the same intensity.

Fixation in alcohol-acetic acid, the subsequent passage through the alcohol series, and hydrolysis with *N* HCl extracts all detectable P^{32} from the cyto-

plasm of these cells at most stages. Two exceptions were noted. Lily microspore mother cells during pachytene incorporate P^{32} in a cytoplasmic fraction that is not extracted by *N* HCl. In well-flattened cells the activity can be definitely identified with the cytoplasm rather than the nucleus. The activity is considerably lower than that which the nucleus incorporates earlier during a similar exposure. Since this incorporation was not found in *Tradescantia*, it probably is not regularly associated with meiosis. A similar retention of activity by the cytoplasm occurs in the maturing pollen of *Tradescantia*. This P^{32} is not completely extracted by either the HCl or the cold and hot $HClO_4$ which is supposed to remove both PNA and DNA. Fortunately the activity is not high enough to interfere with the detection of P^{32} in the nuclei.

At all activities used there was detectable radiation damage as determined by chromosome breakage, but even at the highest activities no disturbance in the timing of phosphorus incorporation could be detected. The amount of P^{32} incorporated could be affected considerably by the concentration of the isotope or by the concentration of carrier phosphate in the solution, but not noticeably by the radiation within the limits used. However, to rule out radiation disturbances, most of the inflorescences were exposed to the solutions containing only 20 μc of P^{32}/ml.

2. *Autoradiographs after Extraction with 1 N Hydrochloric Acid.* This extraction is expected to leave only the DNA phosphorus in the cells. During the period of unsynchronized premeiotic mitoses in sporogenous tissue only an occasional nucleus makes an autoradiograph. As divisions cease and the sporogenous cells enter the premeiotic interphase, there is a synchronized incorporation of P^{32} at what is apparently late interphase (Fig. 3). In lily the incorporation can definitely be placed before the beginning of leptotene of meiotic prophase, but in *Tradescantia* the beginning of leptotene is not easily defined cytologically. Incorporation may extend into early leptotene, but ceases well before the beginning of zygotene which can be readily detected cytologically (17). Very few of the cells of the tapetal layer show activity at this period. No further incorporation has been detected in several series of slides containing all stages of meiosis and the tetrads of microspores. The next period of incorporation precedes the initiation of a recognizable prophase in the division of the microspore nucleus (Fig. 3). In *Tradescantia* the incorporation occurs during the period 24–48 hours before prophase. In lily the period is perhaps 12 hours earlier (bud length of 42–50 mm). Following the microspore division too few stages have been examined to detect further incorporation in lily pollen. A very complete series of *Tradescantia* pollen

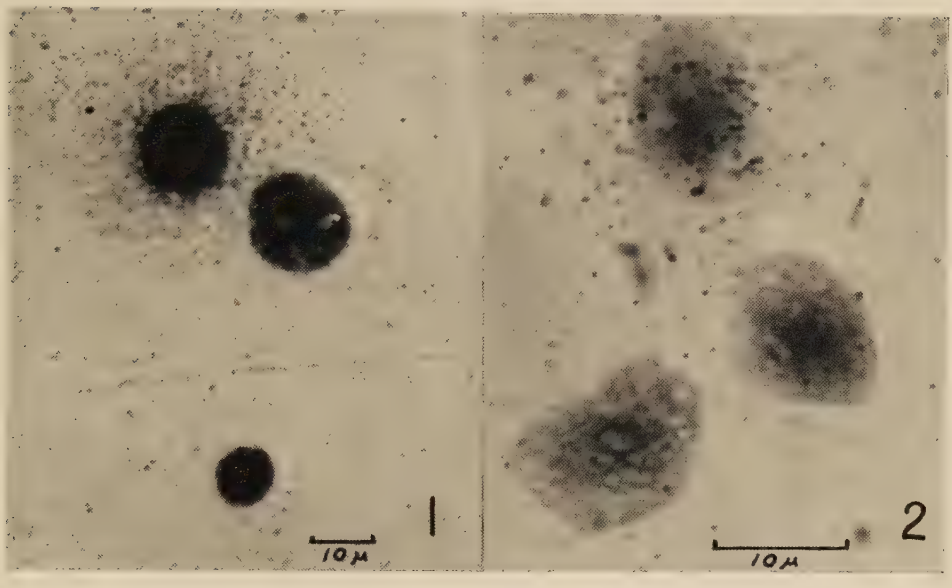

Fig. 1. Tapetal nuclei from a lily anther fixed after the inflorescence had remained 27 hours in a solution containing 54 μc/ml of P^{32}. The upper one has incorporated P^{32} that was not extracted by *N* HCl.

Fig. 2. The nucleus of a tapetal cell at the top has incorporated P^{32} while the other two nuclei of microsporocytes at the pachytene stage do not contain the isotope. The inflorescence remained 10 hours in a solution with 40 μc/ml of P^{32}. Cells were fixed in alcohol-acetic acid and stained by the Feulgen reaction.

stages has shown incorporation in the generative nucleus at the time it begins to assume the elongated condition typical of mature pollen. This may be considered the late interphase since the generative nucleus enters prophase before the pollen is mature. No incorporation has been detected in the tube nucleus, which will not divide again.

Data on the time of incorporation of P^{32} in the tapetal nuclei which surround the sporogenous cells in an anther are incomplete. However, it is noticeable that these nuclei are out of step with the sporogenous cells in their P^{32} incorporation, and yet show a high degree of synchrony among themselves. Nearly every nucleus in this layer undergoes mitosis during the time the sporogenous cells are in pachytene. Soon after the tapetal nuclei pass into interphase, they rapidly incorporate phosphorus. After this period the synchronous behavior is less noticeable. When anthers were exposed for short periods (8 to 12 hours), no nuclei at mitotic metaphase or late prophase contain activity. After longer periods (24–48 hours) in contact with the isotope the metaphase chromosomes as well as interphase nuclei sometimes show

TABLE I

Time of incorporation of P^{32} in sporogenous cells of *Tradescantia*.

Stage during exposure to P^{32} (cells were in contact with the isotope 8 or 24 hours just previous to fixation)	Fixed in alcohol-acetic acid and extracted with 1 *N* HCl	Fixed in cold 70 per cent alcohol and extracted with:		
		Cold 0.2 *N* $HClO_4$	Cold 1 *N* $HClO_4$	Hot 0.5 *N* $HClO_4$
	DNAP retained	PNAP retained[1]	DNAP retained	No NAP retained
Premeiotic interphase (0–2 days) Meiotic divisions (2–6 ½ days)	N (1 ½–2 days)	—	—	—
Prepachytene	none	—	—	—
Postzygotene	none	none[3]	none[3]	none[3]
Microspore interphase (6 ½–11 ½ days)	N (9 ½–10 ½days)	CH (7–8 and 10 ½–11 ½ days)	N (9 ½–10 ½ days)	none
Mitosis in microspore (11 ½–12 days)	none	?	none	none
Interphase in pollen (12–16 days)	N[2] (13 ½–14 days) CL (12–14 days)	CH	N[2] (13 ½–14 days) CL (12–14 days)	CL (12–14 days)

N—activity in the chromosomes.
CH—relatively high activity in the cytoplasm.
CL—very low activity in the cytoplasm.

activity. This confirms the observation (6) that P^{32} is not incorporated during the stages of mitosis.

3. Autoradiographs after extraction with perchloric acid. In this series of slides only anthers of *Tradescantia* were used. Perchloric acid extraction allows the removal of the phosphorus compounds in several fractions (see section on methods). Cells extracted with both cold and hot $HClO_4$ are not expected to contain either PNA or DNA. None of the cells so treated produced a detectable autoradiograph (Table I), except the pollen grains during the interphase 1–2 days after mitosis in the microspores. This activity, which

[1] Applies only when exposed to P^{32} during the periods when DNA does not incorporate the isotope.

[2] Incorporation in generative nucleus only.

[3] Since this article went to press other methods have revealed some incorporation in an unidentified nucleic acid or protein fraction just preceding metaphase I.

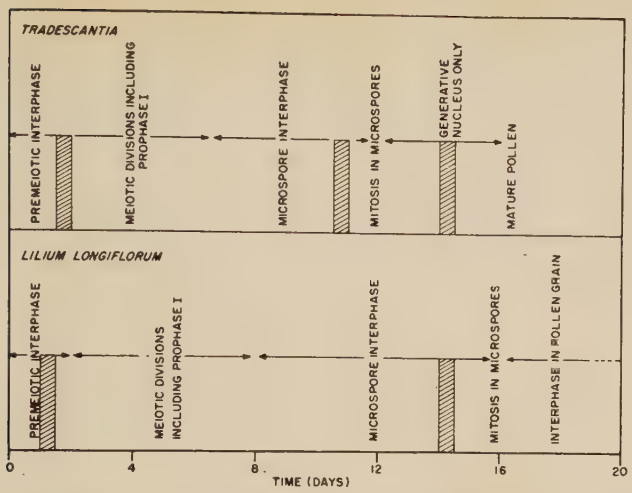

Fig. 3. Chart showing approximate duration of stages in developing sporogenous cells of *Tradescantia* and lily. The shaded areas indicate the periods during which P^{32} is incorporated into the DNA of the chromosomes.

is very low, is in the cytoplasm. It is also present after the extraction with HCl (see p. 166). Perhaps it represents some cytoplasmic phosphoproteins, with a phosphorus-containing moiety more difficult to extract than nucleic acid. Cells extracted with cold *N* $HClO_4$ for 12–16 hours are expected to retain the DNA phosphorus. These cells produced autoradiographs at the same stages as those extracted with HCl (Table I). This indicates that the HCl extraction procedure has about the same effectiveness as the $HClO_4$ procedure in separating various phosphorus-containing compounds from the DNA of cells.

Since phosphorus is incorporated into the DNA of sporogenous cells only during short intervals in the developmental cycle (Fig. 3), the exchange of P^{32} in the PNA fraction can be detected during long periods without interference from activity in DNA phosphorus. Cells exposed to P^{32} during these periods and then extracted with cold 0.2 *N* $HClO_4$ are expected to retain the P^{32} incorporated in PNA. However, these cells give a positive autoradiograph at only certain stages in the developmental cycle. Whether this is regularly correlated with stages of the cell division cycle is not yet clear. No incorporation occurs during pachytene (earlier stages were not tested) or any of the later stages of meiosis, including the period in which the microspores remain together as tetrads. After the microspores separate, there is a short period during which the cytoplasm increases in volume and granules

appear. During this period P^{32} is incorporated in the PNA fraction. Subsequently the granules disappear from the cytoplasm and the increased volume is accounted for by the development of a vacuole rather than an increase in amount of cytoplasm. During this period no incorporation in the PNA fraction occurs. The incorporation begins again before the mitotic prophase (detectable after the incorporation occurs in the DNA), and then becomes very active following the division. During this period of P^{32} incorporation in PNA, the cytoplasm again increases in amount and becomes filled with granules.

DISCUSSION OF RESULTS

The opinion that the amount of DNA increases during prophase and accumulates on the chromosomes as they shorten and thicken persists in spite of considerable evidence to the contrary. Most of the recent evidence indicates that a nucleus and, presumably, a chromosome have a characteristic amount of desoxypentose nucleic acid. This result was first obtained by a biochemical analysis of isolated nuclei (2, 18, 13) and supported by photometric measurements on individual nuclei (11, 15, 16, 8, 1). Photometric measurements (11, 15, 16) further indicated that the amount of DNA increases in the interphase until it is doubled before prophase. At anaphase an equal distribution is made to the tvo nuclei. Since photometric measurements are not useful in the division stages when the chromosomes are condensed, possible variation during mitosis could not be detected. However, the autoradiographic technique shows no incorporation of P^{32} or S^{35} in the mitotic stages of cells in bean roots (6, 7). This result for P^{32} is confirmed for mitotic divisions in *Tradescantia* and lily anthers and extended to include all stages of meiotic divisions in the present report. A critical question is whether increase in amount of DNA is always associated with incorporation of free phosphate. If this is true the failure to find incorporation in division stages is good evidence that DNA does not increase at these times. Evidence indicating a correlation of the two events is the fact that incorporation of P^{32} is not continuous, but is limited to short intervals in the interphase. Additional evidence favoring this view can be obtained by comparison of photometric data on time of increase of DNA in nuclei of sporogenous cells of *Tradescantia* and the time of P^{32} incorporation reported here. Incorporation occurs in late premeiotic interphase and perhaps very early leptotene, in the interphase 24 hours before mitosis in the microspores, and again in the interphase generative nucleus about 2 days after the microspore division. No incorporation was detected in the tube nucleus. These times agree very closely with photo-

metric data (15) on the time of increase of DNA. Doubling of DNA was found at early leptotene, at late interphase in the microspores and only in the generative nucleus during the following interphase. Since the transition from interphase to leptotene in microsporocytes of *Tradescantia* is difficult to detect cytologically (17), the small discrepancy in time of P^{32} uptake and reported increase of DNA at the beginning of meiosis is probably a matter of interpretation of stages. It is significant that the tube nucleus neither increases in DNA content (15) nor incorporates P^{32}. When two quite different methods give such comparable results, confidence in the data as well as the methods is gained.

On the basis of photometric measurements of $HClO_4$ extracts from lily microspores, increase in DNA linear with time was reported for the microspore interphase (9). Superimposed on this increase, which more than doubled the DNA, was an additional rapid increase at what was estimated from bud lengths to be prophase of the microspore division. The total increase would more than quadruple the DNA between meiosis and this mitosis. The rapid increase could be detected also by P^{32} incorporation, but was found to occur in the interphase at least 24–36 hours before prophase. Exposures of lily anthers to the isotope were not long enough to check the linear increase by the autoradiographic technique. However, the 48-hour exposures of *Tradescantia* buds during the microspore interphase should have produced detectable autoradiographs if there is a linear increase in DNA as reported by Bryan (3). No incorporation was detected.

Another interesting correlation of the P^{32} data relates to the time of duplication of the chromosomes. The chromosomes have been assumed to be single at times in the cell cycle when X-rays produce effects which appear as whole chromosome breaks at the succeeding division, while the appearance of chromatid breaks indicates doubleness of the chromosomes at the time of exposure. An extensive series of treatments of buds of *Tradescantia* at 1-hour intervals (14) during the microspore interphase showed only chromosome breaks earlier than 32 hours before metaphase, both chromosome and chromatid breaks in the period 32–26 hours before metaphase, and only chromatid breaks when X-rayed less than 26 hours before metaphase. Earlier data (12) during the same period had indicated the transition from chromosome to chromatid breaks to occur in the interval 48–24 hours before metaphase. Both these estimates of time of duplication correspond quite closely to the period of incorporation of P^{32} and the period of increase of DNA indicated by photometric data (15), if the prophase is assumed to be of relatively short duration.

All these data indicate that the genes are not active during the division stages. Not only chromosome synthesis but other protein synthesis in the nucleus may cease during the division, since it has been shown that the proteins of nuclei decrease during prophase (4). It is perhaps also significant that the P^{32} incorporation in the PNA ceases during the division from metaphase I to the end of meiosis in *Tradescantia*.

A consideration of the available data on the species constancy of DNA per nucleus and the time of its increase as shown by photometric data and incorporation of P^{32} into the chromosomes, leads to the conclusion that a nucleus, or a chromosome, contains a characteristic amount of DNA which is probably part of the permanent framework of the chromosome. There is no evidence from the data of P^{32} incorporation that there is an increase in the DNA during the prophase of either mitosis or meiosis followed by a decrease before the interphase. The apparent changes in optical density and stainability during various stages that give this impression are probably explained by changes in spiralization of the chromonema and by variations in the amount of protein in the nucleus.

SUMMARY

A technique for detection of incorporation of P^{32} into individual nuclei is described. P^{32} is shown to be incorporated into the nuclei or chromosomes during only a relatively short part of the interphase. Evidence that this corresponds to the period of DNA synthesis is presented. Using the evidence on P^{32} incorporation along with the available data on the species constancy of DNA per chromosome set, the time of its increase as measured by other methods, and the time of chromosome reproduction as indicated by X-ray breakage, it is tentatively concluded that the period of P^{32} incorporation corresponds to the time of chromosome reproduction and that the DNA is a part of the permanent framework of the chromosome. It is further shown that incorporation of P^{32} into the PNA of individual cells can be measured by the use of autoradiographs. This incorporation is correlated with periods of rapid accumulation of synthesized materials in the cytoplasm and may in turn be related to the cell cycle.

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IN VIVO AND *IN VITRO* AFFINITY OF DIAMINOACRIDINES FOR NUCLEOPROTEINS[1]

P. P. H. DE BRUYN, R. S. FARR[2], HILDA BANKS, and F. W. MORTHLAND

Department of Anatomy, The University of Chicago, Chicago, Ill.

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It is well known that the cell nucleus can be stained only after the cell has been killed by fixation or other means and that staining the nucleus of the living and undamaged cell is generally not possible. The components responsible for nuclear staining *in vitro*, i.e., staining of the fixed and sectioned material with basic dyes, are held to be the nucleic acids. On cell death the combination nucleic acid-protein is severed or loosened, with the result that basic dyes can combine with the nucleic acids via the free phosphoric acid groups (7). The failure of basic dyes to stain nuclei *in vivo* is usually explained on the basis of the attachment existing between the nucleic acids and the basic groups of the proteins of the nucleus, thus blocking the acid groups of the nucleic acids from combination with the basic dyes.

Among the basic dyes, however, there is one group of compounds which has been shown to have the extraordinary property of staining nuclei *in vivo* as well as *in vitro*. Following parenteral or oral administration of diaminoacridines the nuclei of practically all tissues are stained in the living animal (2, 3). In view of the above mentioned considerations on the interaction of nucleoproteins with basic dyes, the object of the present investigation was to define more clearly the nature of the *in vivo* affinity of the diaminoacridines for nuclei. It is of particular interest to learn whether the staining of nuclear material in the living cell is dependent upon the presence of nucleoprotein and if so, upon which type.

The intranuclear localization of these diaminoacridines is observed only by their fluorescence when stimulated by near ultraviolet light and the presence of these compounds is detected in concentrations which are too low to

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[2] Lt. MC USN. Present address: Naval Medical Research Institute, Bethesda, Md. The opinions expressed in this report do not necessarily represent those of the U.S. Navy.

produce directly visible color. We have considered the possibility that other basic dyes may also enter the living nucleus but in such low concentrations that no visible color is produced. In order to test this, we have investigated whether the *in vivo* affinity of diaminoacridines for intranuclear material is a specific property of the diaminoacridine compounds or whether other basic fluorescent dyes could also be detected in living cell nuclei.

PROCEDURES

To determine the specificity of diaminoacridines with respect to their *in vivo* affinity for nuclei, we examined the following fluorescent basic dyes: berberine sulfate, magdala red, methylene blue, phenosafranine, safranine O, rhodamine B, rhodamine G, rhodamine 6G, and thionine. The dyes were injected subcutaneously in mice in doses varying from 25 mg to 100 mg per kg body weight. The animals were killed with ether one hour after injection. Fresh frozen sections were made of liver, kidney and spleen. Optical procedures were the same as those used for the observation of the nuclear fluorescence by diaminoacridines (2) except that besides the Corning No. 5113 filter, a Corning No. 5874 filter which has a peak transmission at 365 mμ was also used.

In order to obtain information about the nuclear components which are stained *in vivo* with diaminoacridines, we compared the fluorescent staining pattern of a nucleus obtained by *in vivo* administration of diaminoacridines with the staining pattern of the same nucleus after fixing the frozen-dried preparation with methyl alcohol and staining with methods generally used for the demonstration of nucleoprotein, i.e., the Feulgen or the methyl green-pyronin method. The diaminoacridine was proflavine hydrochloride. Rat tissues were used throughout. The technical details involved in the preparation of the frozen-dried sections and other procedures employed for the observation of the nuclear fluorescence by diaminoacridines have been described previously (2). A nucleus was located and photographed. The slide was then placed in xylol which removed the cover glass and dissolved the paraffin-xylol mixture used for mounting the section. The frozen-dried section was then fixed in absolute methyl alcohol for 30 minutes, immersed in 75 per cent and 25 per cent methyl alcohol for several minutes in each solution and finally placed in distilled water. The section was then stained with either the Feulgen or the methyl green-pyronin method. The same nucleus was located and photographed.

In addition, we studied the *in vitro* staining behavior of diaminoacridines on fixed sections in order to see whether their staining behavior corresponded to that of ordinary basic dyes, particularly with reference to their affinity for nucleoproteins. The tissues used were pancreas, lymph node and spinal cord, because of the high ribosenucleoprotein content of their cellular components. The material was fixed in 10 per cent neutral formalin and embedded in paraffin. Since the affinity of basic dyes for nucleoproteins is dependent on the pH of the solution (5) a series of solutions of proflavine hydrochloride of the following pH values was prepared: 2.2, 3.4, 4.6, 5.2, 5.7, 7.0 and 8.0. The sections remained for 4 hours in 1:10000000 solutions of proflavine hydrochloride of the above pH values.

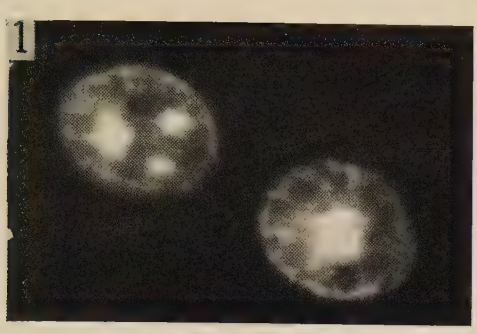
RESULTS

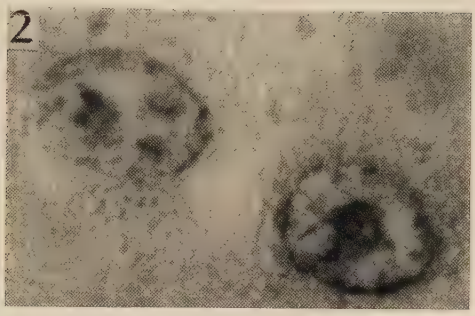
Administration of the other basic fluorescent dyes, listed above, demonstrated that the *in vivo* affinity of diaminoacridines for nuclei is specific for the latter compounds. None of the dyes tested were able to stain *in vivo* the nuclei of any of the tissues examined. It therefore appears reasonable to assume that the diaminoacridine molecule possesses unique and characteristic features enabling it to combine with nuclear material in the living state.

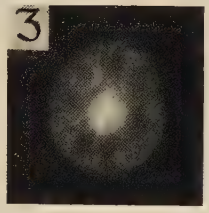
The study to investigate which nuclear components are responsible for this *in vivo* affinity produced the following results: Figs. 1 and 3 show nuclei of a rat liver stained *in vivo* with proflavine hydrochloride. Figs. 2 and 4 show the same nuclei after fixation with methyl alcohol and stained with methyl green-pyronin. Allowing for some minor differences caused by slightly different levels of focusing, the *in vivo* staining pattern of proflavine hydrochloride corresponds exactly with the *in vitro* staining pattern obtained with the methyl green-pyronin method. It must be concluded that the *in vivo* affinity of diaminoacridines for nuclei is based on the content of nucleoproteins. The methyl green-pyronin mixture differentiates between the desoxyribosenucleic acid and the ribosenucleic acid, the former staining green and the latter red. Therefore the nucleolus stains red and is surrounded by a ring of green staining nucleolus-associated desoxyribosenucleoprotein. The diaminoacridines *in vivo* stain the nucleolus as a solid mass and apparently make visible both nucleoprotein components. Essentially the same results were obtained when the Feulgen method was used instead of the methyl green-pyronin mixture. The Feulgen method does not stain the nucleolus itself and the nucleolus in these preparations can only be located by the presence of a ring of nucleolus-associated desoxyribosenucleoprotein. We observed a solid mass of fluorescence at the place where the ring appeared in the Feulgen preparation, indicating that the ribosenucleoproteins within the ring were stained with the diaminoacridine.

The fact that diaminoacridines administered *in vivo* have an affinity for intranuclear desoxyribosenucleoproteins as well as for ribosenucleoproteins raised the question of their affinity for the extranuclear ribosenucleoprotein.

Fig. 1. Two nuclei of rat liver stained *in vivo* with proflavine hydrochloride, 25 mg per kilogram intravenous. Two hours after injection. Frozen dried. Magnification: 1900 ×.
Fig. 2. The same two nuclei as in Figure 1 after fixing the frozen dried section with methyl alcohol and staining with methyl green-pyronin. Magnification: 1900 ×.
Fig. 3. Nucleus of rat liver stained *in vivo* with proflavine hydrochloride, 25 mg per kilogram intravenous. Two hours after injection. Frozen dried. Magnification: 1900 ×.







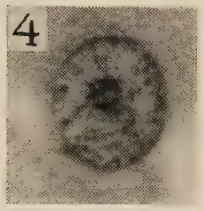


Fig. 4. The same nucleus as in Figure 3 after fixing the frozen dried section with methyl alcohol and staining with methyl green-pyronin. Magnification: 1900 ×.
Fig. 5. Pancreas rat, stained *in vivo* with proflavine hydrochloride, 25 mg per kilogram intravenous. Two hours after injection. Magnification: 750 ×.
Fig. 6. Pancreas rat. *In vivo* staining with 1 : 10000000 proflavine hydrochloride at pH 4.0. Formalin fixation. Magnification: 750 ×.

As reported previously (2), diaminoacridines have *in vivo* a low degree of affinity for cytoplasm. Since the present observations indicate that diaminoacridines administered *in vivo* have an affinity for intranuclear ribosenucleoproteins, it became of interest to examine their behavior towards extranuclear ribosenucleoproteins. For this study we chose the pancreas because of the high content of cytoplasmic ribosenucleoprotein in the cellular components of the exocrine portion of the gland. *In vivo* administration of proflavine hydrochloride results in a faint diffuse staining of the cytoplasm (Fig. 5) but no particular affinity for the extranuclear ribosenucleoproteins at the basal parts of the cells which are easily visualized by *in vitro* staining with ordinary basic dyes. The fact that the nuclei were stained eliminates the possibility that the absence of the *in vivo* staining of extranuclear nucleoproteins is caused by impermeability of the cells for these compounds.

We investigated the possibility that this lack of *in vivo* affinity of diaminoacridines for extranuclear nucleoprotein perhaps also exists in material which is stained with these dyes after fixation. Formalin-fixed pancreas was treated with proflavine hydrochloride solutions of different pH values. Staining took place at all values of pH 7.0 and below, with an optimum of about pH 3.4–4.6. At alkaline pH values, there was a marked reduction of staining intensity, consequently we used acid solutions for the *in vitro* staining with diaminoacridines, choosing a pH of approximately 4.3. The fluorescent pattern of sections stained in this way with proflavine hydrochloride corresponds with the distribution of nucleoproteins as shown by other methods such as ordinary basic dyes, methyl green-pyronin or ultraviolet absorption. The extranuclear nucleoproteins at the basal part of the exocrine cells are distinctly stained (Fig. 6). This was also observed in other cells known to have a high content of extranuclear ribosenucleoproteins, as: lymphocytes, salivary gland, and motor nerve cells. Apparently diaminoacridines have little or no affinity for extranuclear ribosenucleoproteins *in vivo*. They possess like other basic dyes, such affinity *in vitro*.

DISCUSSION

The behavior of diaminoacridines towards nucleoproteins can be summarized as follows: Diaminoacridines have an affinity *in vivo* for intranuclear desoxyribose- and ribosenucleoproteins. There is little or no *in vivo* affinity for extranuclear nucleoproteins. Their behavior *in vitro*, that is staining after fixation, is like that of other basic dyes; they have an affinity for all nucleoproteins regardless of their localization in the cell.

The present state of knowledge of nucleoproteins, particularly in the living organism, does not allow an exact explanation of the exceptional behavior of diaminoacridines as basic dyes toward nucleoproteins. The evidence available at present indicates that the binding between a basic dye and a nucleic acid is a chemical one mediated by the phosphate group of the nucleic acid (7, 8). The nucleoprotein complex is similarly held to be a saltlike combination between the negatively charged groups (phosphate) of the nucleic acid and of the positively charged groups of the proteins (4). The *in vivo* affinity of diaminoacridines for nucleoproteins may be based either on the presence of a number of free phosphoric acid groups or upon their ability to displace the proteins. In this connection, it is of interest to note that Chargaff and Saidel (1), on the basis of their work on nucleoproteins of the tubercle bacillus and their precipitability with lanthanum salts, suggest that free phosphoric acid groups may be available in the undenatured nucleoprotein complex.

The diaminoacridine bases which show the ability to stain nuclei *in vivo* are all strong bases and are thus presumably capable of the displacement reaction. However, the fact that the diaminoacridines have little or no affinity *in vivo* for extranuclear nucleoproteins seems to rule out this mechanism, unless the protein fraction of the cytoplasmic nucleoproteins possesses a higher base strength than those of the intranuclear nucleoproteins. The chemical affinity of the dyes for nucleoproteins must be based in part on the strong basic nature of the dyes. However, the negative results obtained with other basic dyes of the thiazine, xanthine and phenazine series of which methylene blue for example has a base strength comparable to that of 3,6-diaminoacridine, show that this factor is by no means the only one operating.

A strong directing effect may be found in the steric arrangement of the groups in the basic ring structure of the dye. Thus, the amino groups when in the 3 and 6 position exert a strong positive effect on the staining reaction whereas in other positions there is little or no effect, e.g. rivanol with one amino group in the 9 position is only a weak staining agent. In the 3, 6 configuration the two side amino groups are in position to share electrons with the ring nitrogen by resonance thus strengthening the ion forms of the dye. It also appears probable that, with the amino groups in these positions, the structure of the dye molecule assumes a preferred configuration capable of joining strongly with the nucleotides. From the above discussion it would appear as if the spacing of at least two and probably all three of the nitrogen atoms is such as to "fit" the spacing of free phosphoric acid groups of the

nuclear nucleotides. A similar hypothesis is advanced by Kurnick (6) to explain the binding between nucleic acids and certain basic dyes. Perhaps this steric configuration also aids in increasing the ease with which these dyes penetrate the nuclear membrane which excludes other dyes of the required basicity.

Whatever the mechanism of the *in vivo* affinity for nucleoproteins is, their differential behavior towards the intranuclear and extranuclear ribosenucleoproteins points to *in vivo* differences between the two components. These differences exist probably in the availability of the phosphoric acid groups of the nucleic acids, this being greater in the intranuclear nucleic acids than in the extranuclear ribosenucleic acid. A similar difference may exist between the desoxyribosenucleoprotein and the extranuclear ribosenucleoprotein in addition to the difference in the carbohydrate component of their nucleic acids.

SUMMARY

Diaminoacridines have an affinity *in vivo* for intranuclear desoxyribose- and ribosenucleoproteins. There is little or no affinity of these compounds for the extranuclear ribosenucleoprotein. The behavior of diaminoacridines *in vitro*, that is staining after fixation, is like that of other basic dyes: they have an affinity for all nucleoproteins regardless of their localization in the cell. The possible chemical basis for the *in vivo* affinity of diaminoacridines for nucleoproteins is discussed.

The differential behavior *in vivo* of diaminoacridines towards the intranuclear and extranuclear ribosenucleoproteins indicates *in vivo* differences between these two components. Similar differences may exist between the desoxyribosenucleoproteins and the extranuclear ribosenucleoproteins.

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MESSUNGEN DER DOPPELBRECHUNG (Δn) UND DES DICHROISMUS (Δk) AM AMYLOID IN KULTUREN *IN VITRO*

H. H. PFEIFFER

Laboratorium für Polarisationsmikroskopie, Bremen, Deutsche Bundesrepublik

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Fragestellung. — Als im letzten Jahre Fibrocytenkulturen aus Cutis, Aortenwand und Niere des Kaninchens nach der von Paravicini (7) erprobten Technik in Kaninchen-Heparinplasma und Milzextrakt ungeteilt ausgepflanzt worden waren, entwickelten sich im Mutterstück in konzentrischer Anordnung doppelbrechende Strukturen. In dem hart und schwer schneidbar werdenden Mutterstück bestand das anisotrope Material nicht nur aus Reticulin- und Kollagenfasern, wie vor allem im peripheren, zellreichen Rande der Explantate, sondern auch aus unregelmässig durchbrochenen Schalen umwachsenen Plasmas, in deren zentralen, schlecht ernährten Bezirken zufolge der Kongorot-Reaktion auch Amyloid ausgebildet war oder Verkalkung auftrat. Jene histologisch allein dauerfähige Reaktion auf Amyloid spricht zwar auch auf bestimmte hyaline, mucose, fibrinoide und vielleicht elastische Gewebekomponenten an, soll aber nach Ladewig (5, 6) bei Amyloid spezifisch werden, indem sie dort in Kombination mit Anisotropie tritt. Wegen der daraus sich ergebenden eminent praktischen Bedeutung solchen Verhaltens ist eine eingehende polarisationsoptische Analyse, vor allem auch in quantitativer Hinsicht (13), zu fordern: ein Ziel, zu dessen Aufstellung G.-A. Johansson (Stockholm) ein wesentliches Verdienst trägt.[1] Vor gemeinsamer Behandlung weiterer Teilfragen mag hier dazu als ersten Beitrag über die entgegen Ladewig (5, 6) gefundene *Abhängigkeit der Doppelbrechung* (Δn) des Amyloid vom Brechungskoeffizienten bestimmter Imbibitionsflüssigkeiten und über vorläufige *Messbefunde des Dichroismus* (Δk) berichtet werden.

METHODISCHER TEIL

Zur *Darstellung des Amyloid* in den Explantaten ist vor allem die *Reaktion mit Kongorot* ausgeführt worden. Nach der Vorschrift von H. Bennhold sind Gefrierschnitte der Explantate $\frac{1}{2}$ min. in 1 % wässriger Kongorotlösung gefärbt und $\frac{1}{4}$ min.

[1] Für seine Hilfe durch Bereitstellung reichlichen pathologischen und Schnittmaterials zu Vergleichszwecken sei ihm hier nochmals herzlicher Dank gesagt.

in gesättigte wässrige Lithiumcarbonatlösung getaucht, dann in 80 % Alkohol entfärbt und in Wasser gespült, nochmals in Lithiumcarbonat gebracht und 15 min. in Wasser gewaschen und, ohne Nachfärben in Hämalaun, über Alkohol und Xylol in Canadabalsam eingeschlossen worden (Rotfärbung des Amyloid neben ungefärbter Darstellung des Hyalin). Bei der nur gelegentlich ausgeführten *Jodreaktion* sind Gefrierschnitte aus Wasser in stark verdünnte Lugolsche Lösung überführt und nach Auftreten von strohgelber Färbung in Wasser abgespült und in Glyzerin untersucht worden (dunkelbraune Amyloidfärbung). Aus dem positiven Ausfall der *Millonschen Reaktion* nach der Vorschrift von Bensley und Gersh (1) muss wohl auf Vorkommen von Tyrosin enthaltenden Proteinen geschlossen werden, wie auch eine Probe auf Tyrosin (2) beweist.

Die *Messungsanordnung* zur Bestimmung des Δk und der Δn ist in Anlehnung an Zocher und Jacoby (16) zusammengestellt worden. Das durch eine Linse zum Konvergieren gebrachte Licht einer durch Filterung monochromatischen Lichtquelle passiert *nacheinander* den Polarisator, das Objekt, das als Kompensator gebrauchte $\lambda/30$-Glimmerplättchen (9), die Vorrichtung des Drehungshalbschattens, eine verstellbare Blende, die Vorrichtung des Elliptizitätshalbschattens und den Analysator und wird durch eine weitere konvergierend wirkende Linse auf die Lochblende im Okular projiziert. Die beiden konvergierend wirkenden Linsen sind direkt in die Nicolfassungen gesetzt, Polarisator und Analysator drehbar zur optischen Achse eingeklemmt und die beiden Halbschattenvorrichtungen zusammen mit einer Gesichtsfeldblende am Analysator angebracht. Die Vorrichtung des *Drehungshalbschattens* besteht aus zwei 0.06 mm dicken, optisch aktiven Quarzplättchen, welche an ihrer Berührungskante einen feinen schwarzen Querstreifen ergeben und die beiden Gesichtsfeldhälften bei gekreuzter Stellung der Nicols zu übereinstimmender Helligkeit bringen. Die Vorrichtung des *Elliptizitätshalbschattens*, die wie üblich zu voller Wirksamkeit unter 45° zum Analysator gebracht werden muss, ist in Abänderung gegenüber Leiser ein zwischen zwei Federn in seiner Längsrichtung eingeklemmter Glasstreifen, welcher in derselben durch zwei endständige Schrauben in wechselndem Grade gepresst werden kann, und auf den ein sehr dünnes Glimmerblättchen so aufgekittet wurde, dass die Schwingungsrichtungen der vom Glas und vom Glimmer stärker gebrochenen Strahlen vertikal aufeinander stehen.

Um das *Objekt unter 45° zu den Nicols einzustellen*, wird das vorn am Kompensator befestigte Präparat in Verbindung mit dem Teilkreis gedreht, bis die eingeschaltete Vorrichtung des Elliptizitätshalbschattens in beiden Gesichtsfeldhälften übereinstimmende Dunkelheit ergibt, wird die Stellung des Präparats arretiert und nach Lösen seiner Verbindung mit dem Teilkreis dieser allein mit dem zum Kompensieren gebrauchten Glimmerblättchen bis zu maximaler Dunkelheit des Gesichtsfeldes gedreht, schliesslich nach Verbinden aller Elemente das ganze um 45° gedreht, das Präparat arretiert, vom Teilkreis gelöst und dieser mit dem Kompensator in die Ausgangsstellung zurückgedreht.

Zur *quantitativen Ermittlung des* Δk dient die Vorrichtung des *Drehungshalbschattens* (4). Es wird dazu der Polarisator bis zum Erreichen übereinstimmender Helligkeit im gesamten Gesichtsfelde gedreht und der Drehungswinkel ϱ am Teilkreis abgelesen. Wenn, wie bei den hier untersuchten Objekten, die Schwingungsrichtung des Polarisators in Richtung auf die Objektachse (als der Orientierungsrichtung) gedreht werden muss, ist ϱ positiv zu nehmen (und umgekehrt). Sei d die Schicht-

dicke des Präparats, so resultiert (11) der Δk nach den Ableitungen von Zocher und Jacoby (17) zu:

$$\Delta k = \frac{2}{d} \cdot \log \operatorname{tg}\left(\frac{\pi}{4} + \varrho\right).$$

Zur *Bestimmung der* Δn werden nach der Messung des Δk Polarisator und Objekt in die Ausgangsstellung zurückgedreht und der Kompensator in den Strahlengang eingeschaltet. Nachdem dieser zusammen mit dem Objekt um 45° gedreht worden ist, während der Polarisator in die Stellung zur Aufhebung des Δk gebracht wurde, dreht man den Kompensator so weit, dass mit der Vorrichtung des *Elliptizitätshalbschattens* eine rein lineare Polarisation des Lichtes an übereinstimmender Helligkeit der beiden Gesichtsfeldhälften aufgesucht wird. (Manchmal muss zusätzlich auch der Analysator gedreht werden, um die Empfindlichkeit der Einstellung nicht durch die Aufhellung des Gesichtsfeldes infolge der veränderten Schwingungsrichtung des linear polarisierten Lichtes herabzusetzen.) Zur Bestimmung der Schwingungsrichtung des resultierenden linear polarisierten Lichtes wird die Vorrichtung des Elliptizitätshalbschattens aus-, die des Drehungshalbschattens wieder eingeschaltet und dann der Analysator bis zu abermaliger Gleichheit der Helligkeit beider Gesichtsfeldhälften gedreht. Die Stellungen der Teilkreise des Kompensators ($\varkappa$) und des Analysators (α) werden abgelesen und, wenn λ die Wellenlänge des einfallenden Lichtes, ϑ die Δn des als Kompensator gebrauchten Glimmerplättchens bedeuten, nach elementaren sphärisch-trigonometrischen Berechnungen (11, 17) die Δn gefunden zu:

$$\Delta n = \frac{\lambda}{d} \cdot \frac{\sin 2(\alpha - \varkappa) \sin \vartheta}{360 \cos 2\varrho}.$$

Bei dem Ambronnschen *Imbibitionsverfahren zur Bestimmung der Form-(Stäbchen-) und der Eigendoppelbrechung* (10) wird das Objekt mit Flüssigkeiten von stufenweise steigendem Lichtbrechungsvermögen durchtränkt und werden die jeweils gemessenen Intensitäten der Δn (oder die nicht auf konstante Schichtdicke reduzierten Gangunterschiede Γ) als Ordinate in ein Koordinatensystem eingetragen, dessen Abszisse die Brechungskoeffizienten der Imbibitionsflüssigkeiten (n_2) bezeichnen. Mit solcher empirischen Kurve kann die Eigen-Δn bekanntlich aus dem Minimum der Kurve der Gesamt-Δn, die Form-(Stäbchen-)Δn aus der Differenz zwischen totaler und Eigen-Δn bestimmt werden).[1] Ohne dass schon alle Forderungen an ideale Imbibitionsmedien erfüllt werden konnten, haben sich doch unter Berücksichtigung der zahlreichen Fehlerquellen (10) des Verfahrens reproduzierbare Kur-

[1] Zwar bezieht sich die Wienersche Theorie, indem sie aufgrund der Dielektrizitätskonstanten $\varepsilon = n^2$ abgeleitet wurde, auf die *Quadrate* der Brechungskoeffizienten, doch ist die Abweichung im experimentellen Bereiche von $n = 1$ bis 2 gering. Um aber die Kurven in diesem nicht weithin mit der x-Achse zusammenfallen zu lassen, werden die empirischen Werte in meist vielfacher Überhöhung aufgetragen. Bei den so resultierenden *affinen Hyperbeln* ändert die Asymptote x ihre Richtung nicht, verläuft aber die Asymptote y wesentlich steiler und geht die Symmetrieachse der Hyperbel, als Bisektrix der beiden Asymptoten, nicht durch den affinen Punkt des ursprünglichen Scheitels, sondern nähert sich der Scheitelpunkt jenem Punkte, bei welchem die x-Achse die Hyperbel als Tangente berührt.

ven bei Gebrauch von Mischungen aus Cyclohexanol ($n_2 = 1{,}4645$), Benzylalkohol ($n_2 = 1{,}5376$) und Schwefelkohlenstoff ($n_2 = 1{,}6276$) ermitteln lassen. Insbesondere zu empfehlen sind diese Mischungskomponenten, da bei ihrem Gebrauch quellende Wirkungen auf die Mischkörper und damit herrührende Desorientierungen ihrer Leptonen fast ganz ausgeschlossen bleiben und eine beabsichtigte experimentelle Veränderung des Lückenvolumens der Mischkörper (12) nicht von den Imbibitionsmedien durchkreuzt oder gestört wird.

BEFUNDE UND DISKUSSION

1. Bei kompensatorischer Messung der Δn erhaltene Werte streuen beim Amyloid *ungefärbter Gefrierschnitte* in schwer verständlicher Weise von mehr oder minder deutlicher Isotropie bis zu schwacher Anisotropie. Das gilt ebenso für Explantate aus verschiedenen Organen wie für solche übereinstimmender Herkunft. Mit Kongorot *gefärbte Gefrierschnitte* sind hingegen stets positiv doppelbrechend, wenn auch in der Intensität etwas variabel je nach dem verschiedenen Quellungsgrade des Amyloid (s. unten). Entgegen Ladewig (5, 6) zeigt das Amyloid gefärbter Schnitte auch nicht eine Zunahme der Intensität der Δn symbat vermehrter Farbstoffdarbietung. Selbst wenn Explantate derselben Herkunft nach wechselnd abgestufter Farbstoffspeicherung verglichen werden, ist eine *direkte* Proportionalität zwischen Farbstoffkonzentration und Intensität der Anisotropie nicht sicher nachweisbar. Aus solchen und ähnlichen Befunden ist gewiss zu folgern, dass das Amyloid in den Gefrierschnitten *nicht erst durch Kongorotaufnahme optisch anisotrop* wird. Eine andere, von Schmidt (14) für kongorotgefärbte Chitinsehnen diskutierte, Deutung kann die Isotropie ungefärbten Amyloids nicht finden, da Amyloid nicht negativ-eigendoppelbrechend auftritt.

2. Wenn Versuchsreihen nach der Ambronn'schen *Imbibitions*methode durchgeführt werden, ist ausserdem in Abweichung von andern Beobachtungen (5, 6) am Amyloid der Explantate *in vitro* keine Unabhängigkeit vom Brechungskoeffizienten n_2 des Imbibitionsmediums festzustellen. Wenn in der Literatur gegenteilige Befunde verzeichnet werden, muss wohl angenommen werden, dass die Wahl der Imbibitionsmedien ungeeignet war, d. h. ihr Permeationsvermögen bei dem Objekte nicht ausreichte. Mit als geeignet erkannten, den vielerlei Anforderungen besser entsprechenden Durchtränkungsflüssigkeiten ergeben die empirischen Bestimmungen der Gesamt-Δn bei affiner Eintragung und 10^3-facher Überhöhung der Ordinatenwerte gut reproduzierbare *hyperbolische Kurven* der erwarteten Krümmungsrichtung, aus deren Lage im Koordinatensystem sogleich auf das Vorkommen von *positiver Stäbchen- und* ziemlich schwacher *positiver Eigen-*Δn geschlossen

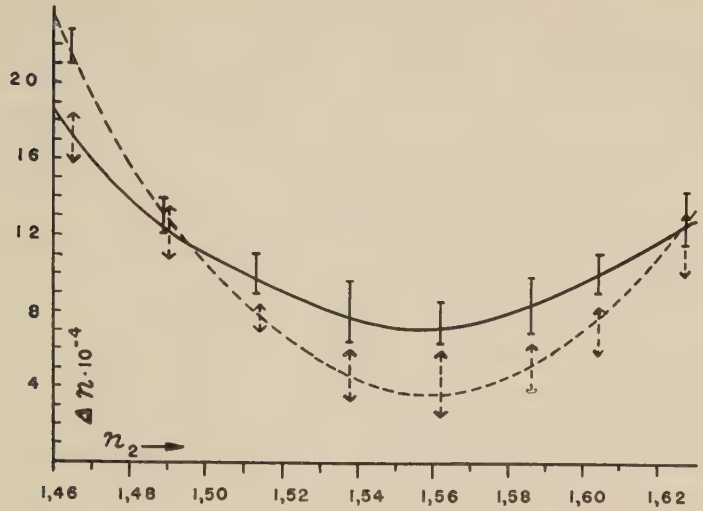

Fig. 1. Gesamtdoppelbrechungskurven des Zusammenwirkens von positiver Form- und positiver Eigen-Δn am Mischkörper des Amyloid, aufgenommen nach Messungen bei Gebrauch einer Cd-Spektrallampe und a) ——— Filter RG 1 Schott & Gen. ($\lambda = 6438{,}5$), b) - - - - - - Filter Agfa 43 ($\lambda = 4678{,}2$); vgl. PFEIFFER: *Z. wiss. Mikrosk.* **60**, 149 (1951).

werden muss (Abb. 1). Der Ordinatenwert des Kurvenminimums (3,5 bis $7 \cdot 10^{-4}$) gibt die Eigen-Δn, der Abszissenwert dieses Fusspunktes der Kurve ($n_2 = 1{,}55_6$) das Lichtbrechungsvermögen des Amyloid an. Geringe Streuungen der Werte der Eigen-Δn in verschiedenen Versuchsreihen auch bei Explantaten derselben Abkunft sind mit Differenzen des Quellungsgrades in quantitativ einleuchtender Weise erklärbar. Durch *Quellwirkung* beim Imbibieren kann nicht immer ganz vermieden werden, dass das Amyloid Wasser aufnimmt, so die Micellbildung gefördert wird (?) und dadurch eine erhöhte Eigen-Δn hervorgerufen wird.

3. Wie bei andern Wiener'schen Mischkörpern (12) kann auch am Amyloid aus der Lage der Kurven der Gesamt-Δn im Koordinatensystem und aus ihrem Krümmungsgrade auf den *Quellungszustand* des Mischkörpers bezw. auf die Quellwirkung der Imbibitionsflüssigkeiten und den *Volumanteil der Interstitien bezw. der Stäbchen* des Mischkörpers geschlossen werden. Mit fortschreitender Reduktion der Lücken-Volumina zeigt sich das Anwachsen der Eigen-Δn in einer vertikalen Verlagerung des Fusspunktes der Kurven, der An- und Abstieg der Stäbchen-Δn in zu- und wieder abnehmender Durchbiegung der Kurven und ihrer endlichen Entartung zu einer Geraden, welche von der Abszisse gerade den doppelten Abstand aufweist im Vergleich zum

Fusspunkt der Kurve stärkster Durchbiegung (12, 13). Sei beim Quellungsgrade „1" das Lückenvolumen $\delta_2 = 0$, so lässt sich aus den empirisch ermittelten Quellungswerten q für die einzelnen Kurven ein wahrscheinlicher Wert δ_2 berechnen. Setzt man beispielsweise für Benzylalkohol die Quellwirkung „1", was mit allergrösster Annäherung wohl zutrifft, so resultiert als Lückenvolumen für Amyloid der explantierten Aortenwand mindestens 0.09 oder der *in vitro* kultivierten Cutis etwa 0.07.

4. An histologischen Materialien kommen zwar auch nicht-dichroitische Färbungen mit Kongorot vor, aber in Bestätigung zu Ladewig (5, 6) und andern Beobachtern zeigt die Kongorotreaktion des Amyloid gut messbaren *Dichroismus* Δk, d.h. parallel und vertikal zur Achse der Leptonen unterschiedliches Absorptionsvermögen. Bemerkenswert ist dabei, dass der dichroitische Farbumschlag in diesen Fällen nicht wie sonst rot—farblos, sondern gelb—grün ist. Wie in einer vorläufigen Prüfung dazu festgestellt werden konnte, bedeutet das für Wellenlängen um $\lambda = 5900$ Å das Ausbleiben von Dichroismus, für solche um $\lambda = 5400$ Å einen Dichroismus positiv zur Länge. Diese Verschiebung des Dichroismus in den Spektralbereich kürzerer Wellenlängen mag auf einem Einfluss der Amyloidsubstanz als Substrat beruhen und einem Effekt des Lösungsmittels von Farbstoffen auf deren Dichroismus (18) vergleichbar sein. Einige vorläufige *vergleichende Messungen* der Δn und des Δk bei bestimmten Wellenlängen *über den gesamten Spektralbereich* des sichtbaren Lichtes hinweg haben die erwartete Bestätigung der Babinet-Zocher'schen Regel ergeben, d. h. auch in den vorliegenden Fällen wird im langwelligen Spektralbereiche der stärker absorbierte Lichtstrahl stärker, im kurzwelligen aber schwächer gebrochen (11, 15).

5. Nach dem Stande der Versuche ist es noch nicht möglich, zu der Frage nach der *Natur des Amyloid* erneut Stellung zu nehmen; das bleibe daher der Fortsetzung der Untersuchungen in Gemeinschaft mit Johansson vorbehalten. Immerhin führen die Beobachtungen an den glasig homogenen Ablagerungen des Amyloid nach der durch Messungen der Δn und des Δk nachgewiesenen Anisotropie zu der Folgerung, dass wir die Entstehung dieses in pathologisch gestörter Biosynthese gebildeten Proteins sehr wahrscheinlich auf ein zeitliches Zusammentreffen von gewissen Kondensations- *und Orientierungs*prozessen zurückzuführen haben. Es genügt offenbar nicht nur ein Erstarren (eine Gelbildung) des durch Aderwände hindurchtretenden emulgierten Amyloidsols (3) als Beginn der Amyloidablagerung anzunehmen, sondern es scheint wesentlich zu sein, dass dabei eine durch die verschiedenen Messungen der Anisotropie erwiesene *Orientierung der Proteinleptonen* hinzutritt. Angenäherte Berechnungen mittels einer bereits mitgeteilten

Methode (8, 12) haben auch zu approximierten Werten des *Orientierungsgrades* (je nach Ausbildungsmodus und -zeitdauer 5 bis 18 % der beteiligten Leptonen) geführt, welche grössenordnungsmässig mit Befunden von andern Proteinen gut in Einklang stehen und somit unser Vertrauen verdienen.

SUMMARY

The *amyloid* within certain explants of rabbit tissue fragments was studied with respect to its *dichroism* (Δk) and its *birefringence* (Δn) in dependence on the refraction of different imbibition media. *Micromethods* suitable for quantitative measurements of Δn and Δk were given, *some dates* with regard to intrinsic birefringence, the light refraction of amyloid, the volume of intermicellar spaces of the rodlet composite body, and the orientation rate of its leptonic rodlets were pointed out, and some of the *implications* of the findings were discussed.

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A METHOD OF FREEZE-DRYING FOR ELECTRON MICROSCOPY[1]

R. C. WILLIAMS

University of California, Virus Laboratory, Berkeley, Calif.

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In the ordinary practice of electron microscopy of biological objects the most serious source of structural deformation of specimen material is the force of surface tension, operating with greatest effect during the final stages of drying of the specimen. As a consequence of this force there are principally two types of structural alteration: a flattening of particulate objects such as cells and viruses, and a clumping and aggregation of the particles as they dry on the specimen film. As a consequence of the prevalence of these artefacts the electron microscopist seldom believes what he sees; his sight tells him, for example, that the larger viruses are oblate ellipsoids, but he systematically and deliberately concludes from this visual evidence that the same viruses are really spherical. The severe flattening of the larger viruses, such as influenza virus, renders a determination of particle diameter ambiguous, since the dependence of apparent diameter upon the degree of flattening is not easy to evaluate. The clumping of particles during drying can easily lead to false impressions concerning the nature of particle association, such as that between bacteria and bacteriophage.

Attempts have been made to reduce the magnitude of the structural artefacts due to drying by the development of two very different methods. The older method, commonly termed "freeze-drying", is a well-established technique for the preservation of biological structure at all levels of size. The details of the method which is at present used in electron microscopy have been described by Wyckoff (7). The results of the application of this freeze-drying technique, however, have appeared to be generally disappointing, as evidenced by the rarity with which the method is used and by the similarity in appearance between published micrographs of normally prepared specimens and similar specimens when frozen-dried.

A more recent approach to the preservation of three-dimensional structure of small biological objects has been made by Anderson (1). This technique avoids the structural artefacts of drying by causing the specimen material to "dry" out of CO_2 at a temperature above its critical point. The published

[1] Aided by a grant from the National Foundation for Infantile Paralysis.

results are beautiful indeed, and micrographs of such delicate objects as human red-cell ghosts and paramecia show what is apparently an excellent overall preservation of structure. The difficulties inherent in the technique have been detailed by Anderson; they are primarily the likelihood of chemical and physical changes brought about by the introduction of the specimen into solvents (alcohol, amyl acetate, liquid CO_2) which are far from physiological. Other drawbacks inherent in the critical point method are that the specimens must always be observed in a chemically fixed state, and that the specimens usually cannot be shadowed, since the preparative technique frequently requires that they be trapped between two films of collodion.

The disappointing results obtained from current freeze-drying techniques indicate strongly that considerable improvement should be made in the specific application of the method to the electron microscopy of suspensions of particulate objects. There are certainly unique advantages potentially associated with the preparation of specimens of this type by freeze-drying: either fixed or unfixed material can be used, no foreign chemical environments are experienced by the specimen, and the specimen material is instantly frozen into a solid matrix, thus tending to preserve intact the structural relationships existing in the normal aqueous suspension.

GENERAL REMARKS ON FREEZE-DRYING

A completely satisfactory method of freeze-drying particulate suspensions for electron microscopy should encompass several desirable aspects, outlined as follows:

1) The aqueous suspension should be frozen and brought to a temperature well below the freezing point as quickly as possible. It is particularly important that the change in temperature in the vicinity of 0° C be rapid.

2) The vacuum sublimation of the volatile components (mostly ice) should be carried out at as low a temperature as possible, consistent with the time that can reasonably be allowed for the sublimation process. The sublimation temperature must be below the freezing point of any eutectic concentration which may develop during the sublimation.

3) The ice should be sublimed as completely as possible before the temperature is raised, and the specimen should be at a temperature several degrees higher than room temperature before it is exposed to the air of the laboratory. The latter step effectively retards condensation of moisture upon the dry surfaces of the specimen material.

4) The examination of the frozen-dried material is facilitated if it is well spread out upon the specimen film, rather than clumped in large and opaque masses. It is particularly desirable to have small specimen particles (and any accompanying polystyrene latex particles) displayed in the form of droplet patterns (2), so that precise qualitative comparisons and quantitative counts can be made.

CRITICAL EVALUATION OF THE CURRENT PRACTICE OF FREEZE-DRYING FOR ELECTRON MICROSCOPY

Wyckoff (7) has described a method of freeze-drying which is appealing in its simplicity, but which can be shown to be inadequate. In this method, some collodion-coated microscope grids are first cooled by placing them on a block of chilled metal. Large drops or micro-droplets of the suspension to be frozen-dried are then placed on the cooled grids. Immediate transfer is made to an ordinary vacuum system (shadow-casting unit), and pumping continued until the block has come to room temperature. The dried specimen on its supporting film is then shadowed in the same vacuum.

The above-described technique can provide for only a relatively small rate of cooling of the specimen drops. When ordinary resting contact is made between two solids (such as a specimen grid on a copper block) the surfaces form molecularly close contact at only three points. This degree of contact provides negligible thermal transfer, and hence the rate of cooling depends upon radiation, and upon air convection and conduction. The last two effects are the most important in the present case, where the average thickness of the air layer between the collodion-coated grid and chilled block are used, even though the bulk of each is pipetted off immediately, a water layer at least 100 μ thick must remain. If it is assumed that a temperature difference as great as 210° C initially exists between the chilled block and the drop of suspension, and that most of the thermal conductivity is through the air layer, the initial cooling rate of the upper surface of the water drop can be calculated. The approximate rate is 300° C/sec, and the time taken for the drop to pass through the change of phase from water to ice is of the order of 0.3 sec. As will be seen later on, this is a very low cooling rate indeed.

The inadequacy of this method of preparation is even more apparent when one considers the temperatures existing in the frozen drop during vacuum sublimation. Only the last stages of the sublimation need be considered, when the heat capacity of the residual ice is very small, since this is the most important part of the sublimation cycle. At this stage the non-volatile material has been concentrated upon the surface of the residual ice layer, and if the temperature of the residual layer reaches the melting point, no true freeze-drying will result.

The vacuum sublimation cycle, in the currently practised technique, is performed in an ordinary bell-jar at room temperature. The thermal transfer conditions are now very simple; there is negligible conduction through the residual air, and as has been pointed out above, there is negligible conduction

through the points of contact between grid wires and chilled block. The thermal equilibrium existing at the end of the sublimation will be established by radiative transfer alone, and the temperature of the sample can readily be estimated by an application of Stefan's law. If T_c is the temperature of the cold block, and T_a is the temperature of the bell-jar (ambient room temperature), then T_s, the temperature of the specimen, is given by: $T_a^4 - T_s^4 = T_s^4 - T_c^4$. This relation holds because the solid angles subtended at the specimen by the cold surface and by the room-temperature surface are equal, and because the emissivity of the chilled metal block and the metallized vacuum bell-jar will be nearly the same. Hence, $T_s^4 = (T_c^4 + T_a^4)/2$. If the chilled block is initially cooled to —190° C its temperature might be as low as —100° C when the ice is finally sublimed. If $T_a = 25°$ C, then $T_s = -13°$ C. If, as has been described in the literature (7), the block is initially chilled with dry-ice only (—78° C), and warms to approximately —50° C at the end of the sublimation, then $T_s = -3°$ C. It is evident that the latter temperature is not low enough for proper sublimation, and even the former one can hardly be expected to be adequate if the residual ice approaches the eutectic concentration of any dissolved electrolyte.

A NEW METHOD OF RAPID FREEZING AND SUBLIMATION

A. A method has been developed for freeze-drying electron microscope specimens which embodies the four *desiderata* outlined above. In this method provision is made for spraying micro-droplets of suspension directly upon a collodion-coated, chilled, copper surface, and for subliming the flattened, frozen droplets at a low and controlled temperature.

The apparatus is shown in Fig. 1. The freezing and sublimation procedure is as follows: A collodion film is placed upon the smooth surface of the copper support. This is readily done by first forming a collodion film on a small ring, and then placing the ring over the support. Intimate contact between collodion and copper is achieved by moistening the collodion with breath moisture at the time it is placed on the copper. The copper support is then lowered into the glass sublimation tube, and stuck to the bottom of the tube with a patch of "DC High Vacuum Grease". This step is necessary in order to assure good thermal contact between glass and copper and thus allow the temperature of the copper to be easily controlled. The open end of the glass tube is then covered, and the tube surrounded by the cooling liquid (liquid N_2, or a CO_2–alcohol mixture). The copper support is brought to equilibrium temperature in about five minutes.

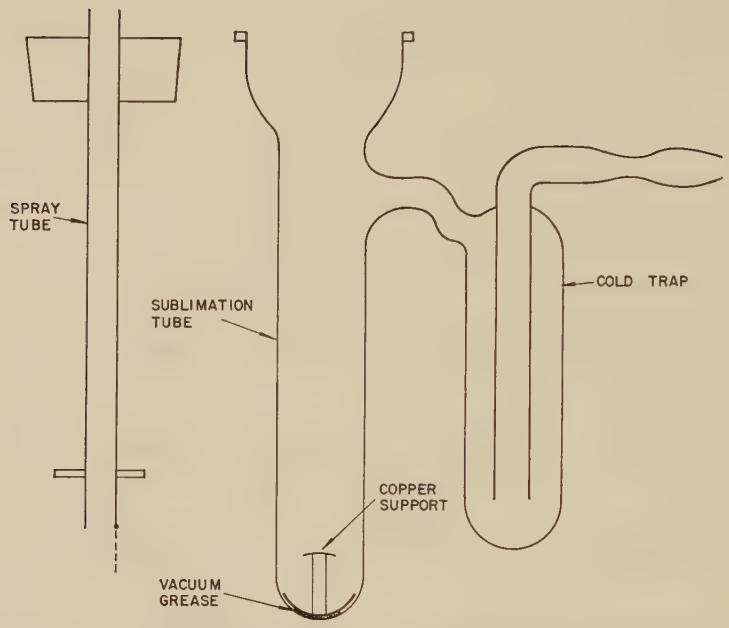


Fig. 1. Drawing of assembly in which spray drops are frozen upon impingement, and vacuum sublimed. Separate spray-tube is shown, with rubber guides for centering. Copper support is pre-filmed with collodion.

A spray-tube (shown separately in Fig. 1) is then inserted into the cold glass tube and a small quantity, of the order of 0.01 ml, of the suspension sprayed upon the collodion-coated copper surface. The spray-tube is then removed, a vacuum-tight cover (with rubber gasket) is placed over the end of the cold tube, and the tube and trap evacuated to less than 10^{-4} mm of mercury. During the sublimation the trap is cooled with liquid nitrogen and the sublimation tube surrounded with a bath at about —50° C. An adequate sublimation time is 15 minutes. At the end of this time, the tube is placed in a water bath at 70° C for 10 minutes. This treatment raises the temperature of the copper surface to about 50° C. Air is then slowly admitted through a stop-cock in the cover.

After the air is admitted the copper support is removed from the tube, the silicone grease is cleaned from its under surface, and it is placed in an ordinary shadowing unit. The collodion film must now be transferred to specimen grids for electron microscopy. A two-step transfer is necessary, each in the manner of a replication, in order to bring the specimen objects

on the "right side" of the grid wires: that is, on their lower side as mounted in the electron microscope. The first transfer is made by use of a piece of Scotch tape in which there have been punched two holes about 3/16 inches in diameter. The piece of tape is placed gently upon the collodion-coated surface and is pressed into close contact around the edges of the holes. When the tape is pulled away the collodion film comes with it and is stretched across the holes, effecting the first transfer. Another strip of Scotch tape is now placed upon a piece of glass, adhesive side up. (This strip of tape is held flat with the aid of a piece of double-coated tape, previously placed upon the glass.) Two 200-mesh grids are placed the proper distance apart upon this second strip of Scotch tape, with a piece of paper about 1 mm square between each grid and the tape. The first piece of tape is now placed, adhesive side down, upon the second piece of tape, with the holes centered over the grids. The collodion film in the space between the 1/8-inch grids and the 3/16-inch holes is gently pressed into contact with the tape by means of a sharpened orange-wood stick. The grids can then be removed in the usual way with a pair of tweezers, and are ready for observation in the microscope.

The deposition of the micro-droplets can be accomplished with either a low-velocity spray from a commercially-available nebulizer[1] or from a high-velocity gun (2). The former is preferable if the suspension contains relatively large objects, such as cells, since the risk of damaging the objects by high-velocity impact is reduced. Such droplets freeze before they splash to large diameter, however, and the observed droplet pattern is not well spread out. If small objects, such as viruses, are sprayed, the high-velocity gun is preferable, since the force of impact causes the droplets to spread out into a thin layer of large diameter before freezing occurs. This facilitates uniform sublimation of the ice, and results in a droplet pattern containing well-dispersed particles.

If a low-velocity spray is deposited, a difficulty associated with the electrical charging of small droplets is encountered. The droplets emerge from the bottom of the spray-tube with a net electrical charge with respect to the tube, and deliver their charge to the copper support as they impinge upon it. The resultant electrostatic repulsion effectively prevents the collodion-coated copper from becoming adequately covered with specimen material. This difficulty is avoided by using a metal spray-tube, and electrically connecting the tube and the copper support with a length of very small brass chain.

[1] Specified as "Vaponefrin Vaporizer", obtainable from the Vaponefrin Co., Upper Darby, Pa. It should be modified by the addition of a short, 90° extension at its exhaust end.

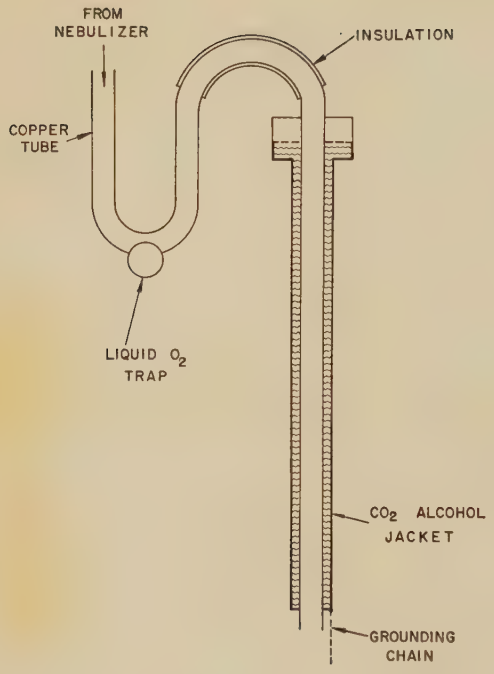
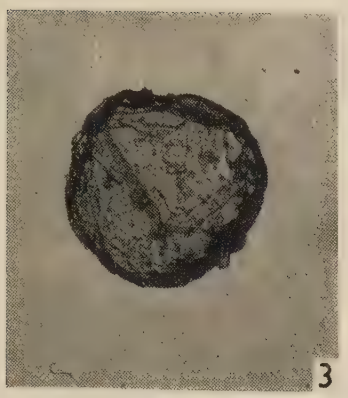


Fig. 2. Drawing of spray-tube in which droplets are frozen in flight. Vertical portion fits into sublimation tube shown in Fig. 1.

When the high-velocity spray is used, the "grounding" of the copper surface is not needed, probably because of the high velocity of droplet impingement.

B. An alternative method of freeze-drying has been developed in which the micro-droplets are frozen in flight and are made to impinge upon cold collodion-filmed grids. This method has the theoretical advantage that there is no period during which any of the suspended biological material can come in contact with, and possibly adsorb to, the wet collodion substrate. It has the theoretical disadvantage that the rate of freezing of the droplets is probably not as great as that provided by method *A*. The supposed lower rate of freezing, however, may not actually be a fact, since relatively pure water droplets of very small dimensions (<10 μ diameter) are known to supercool to a temperature as low as —40° C.

Figs. 3–7. Micrographs of frozen-dried human red-cell ghosts.
Fig. 3. Unshadowed, unfixed specimen.
Fig. 4. Micrograph obtained with polarization microscope, showing strong birefringence.
Fig. 5. Unshadowed, fixed specimen.
Fig. 6. Shadowed, unfixed specimen.



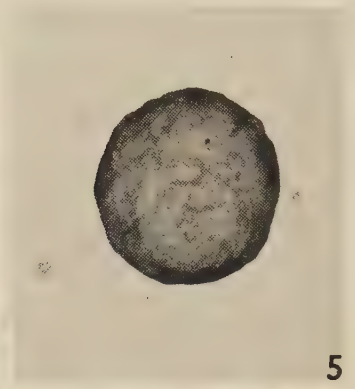
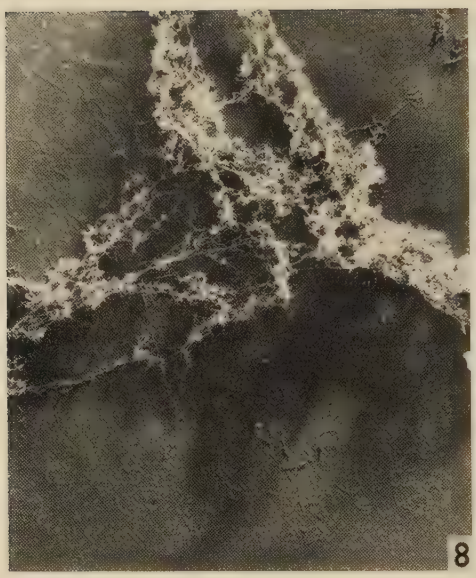


Fig. 7. Shadowed, fixed specimen. Unfixed specimens exhibit some distortion, while the fixed specimens (as shown by the shadows) are almost perfectly spherical. M = 4000 × for electron micrographs.



Figs. 8–9. Frozen-dried tobacco mosaic virus.
Fig. 8. Low magnification micrograph (8000 ×) of portion of droplet pattern of concentrated suspension. The three-dimensional structure is demonstrated by the very elongated shadows.
Fig. 9. Portion of Fig. 8 at increased magnification (50000 ×). Arrows point to TMV particles which are in contact with the substrate at only their ends.

The apparatus for method *B* is like that of method *A*, except for two modifications: collodion-filmed grids are simply held to the copper support with double-coated Scotch tape; the spray-tube must be of copper and should have provision for being cooled with as cold a liquid as feasible, such as liquid nitrogen. Fig. 2 shows a form of spray-tube which has proven satisfactory for use with liquid nitrogen. The U-tube portion is placed in liquid nitrogen, while the jacket around the straight portion is filled with CO_2-alcohol. This division of cold and cool portions is made ncecessary by the fact that the oxygen of the air is liquefied in a tube cooled by liquid nitrogen. A trap is shown in the U-tube to catch the liquefied oxygen and hence prevent it from clogging the tube. If the straight portion of the tube is cooled with liquid nitrogen, descending liquid oxygen will soon cover the specimen screens. In this apparatus the spraying is done with a low-velocity nebulizer, since the droplets are to be frozen during their flight through the cold portion of the tube. In both methods the lower end of the straight spray-tube is placed about 2 cm from the collodion film.

RESULTS

Anderson has found that a very sensitive test object for testing the excellence of his critical-point drying method is the hemolysed human erythrocyte. The membrane thickness of such "ghosts" is only about 10 mμ, and the slightest force of surface tension upon drying will flatten the object completely (3, 4). It was thought desirable to use red-cell ghosts in connection with the present development in order to facilitate a comparison with the results of the critical-point method.

Human red-cell ghosts were prepared by dropping small amounts of fresh blood into a large volume of water. The cell membranes were then lightly sedimented and the supernatant fluid discarded. Fixed ghosts were prepared by taking up the first centrifuged sediment in 1 per cent osmic acid. Unfixed ghosts were also prepared, but only with difficulty; examination of suspensions in the phase contrast microscope showed that the ghosts were very liable to partial destruction during the washing process. It is appreciated that the preparation of the ghosts would have been facilitated by washing in physiological saline following the original lysis, but it was considered necessary to eliminate all non-volatile solvent material before freeze-drying in order not to obscure the details of the cell structure.

Figs. 3–7 show some results obtained with fixed and unfixed red-cell ghosts as frozen-dried by method *B*. (Method *A* produces identical-appearing ghosts.) It is seen that overall structure is best preserved with the fixed cell membranes, although even here there seems to be an area of flat contact between membrane and collodion film. It is believed that the micrographs of the fixed cells show a preservation of three-dimensional structure which compares favorably with that exhibited by the critical point technique.

The unfixed cell membranes show some degree of deformation of structure, but this deformation may have occurred during the preparation of the cell membranes in distilled water. A greater *detail* of surface structure is apparent in the unfixed cell membranes, where a network of small holes is seen all over the membrane surface. In the fixed membranes these holes are not evident, and the whole surface appears to be smoothed. Stereograms of the fixed membranes show interior intertwining filamentous material, but the unfixed membranes do not show this.

In Fig. 4 is also shown a photograph of a fixed red-cell membrane obtained with crossed Nicols in a polarizing microscope. A high degree of birefringence is indicated, far greater than red-cell membranes show when in water (6).

The sign of the birefringence of the membrane material is negative with respect to the radius of the cell.

Figs. 8–9 show results obtained from another sensitive test material, a concentrated suspension (0.01 per cent) of tobacco mosaic virus, prepared by method *A*. The preparation was shadowed at a 2/1 angle, and the small-scale micrograph shows strikingly the three-dimensional aspect of the randomly arranged linear particles. The enlarged micrograph shows that some of the single particles are supported by the substrate in an almost end-on position.

DISCUSSION

A. *Freezing rate.* — A rough calculation can be made of the rate of cooling of the high-velocity droplets impinging upon the collodion-filmed copper surface. There are two primary uncertainties in the calculation: the excellence of contact between collodion and copper, and the degree to which the splashed water droplet is in true liquid contact with the collodion. If it is assumed that both of these contacts are perfect, then the initial rate of heat transfer across the spread-out water droplet can be expressed as:

$$Q/t = \frac{C\,A\,\Delta\,T}{d}\text{, where}$$

C = thermal conductivity of water.
A = surface area of droplet.
d = thickness of droplet.
ΔT = temperature difference between top and bottom of droplet.

(The resistance to thermal flow of the film of collodion only 0.1 μ thick is negligible compared to a layer of water 2–3 μ thick.)

Measurements of typical frozen-dried droplet patterns show that the droplet volume is about 10^{-9} ml, the area is about 4×10^{-6} cm², and the thickness is consequently 2.5×10^{-4} cm. ΔT is taken initially as 185° C, since the upper surface of the droplet is slightly below room temperature, while the surface in contact with the copper will be at about —170° C. Using these numerical values, $Q/t = 4 \times 10^{-3}$ cal/sec. The rate of change of temperature will then be $\frac{4 \times 10^{-3}}{10^{-9}} = 4 \times 10^{6}$ degrees/sec.

A more realistic calculation might be obtained by assuming that some air exists between collodion and copper. With a thin collodion film, however,

no interference colors are seen resulting from this presumed air layer, and electron microscopic observation shows that the film faintly replicates the fine polishing scratches on the copper surface. It is unlikely that the presumed air layer is thicker than 0.2 μ. It is probably also realistic to assume that a film of water vapor initially exists between the lower surface of the droplet and the surface of the collodion. A reasonably generous guess as to the thickness of this layer would be 1.5 μ, half the thickness of the spread-out droplet. A recalculation of the initial rate of change of temperature, based on these two estimates, yields a value of 2×10^5 degrees/sec.

A more significant calculation is the time taken for the droplet to pass through the liquid-solid phase change. It is probably correct to assume that the water layer has come into true liquid contact with the collodion surface by the time freezing has started. A calculation shows that the upper surface of the water droplet (the last layer to freeze) will pass through the change of phase in the order of 10^{-5} sec. Even if the copper surface is cooled only to —78° C, the freezing time will still be very brief, of the order of 2×10^{-5} sec. A comparison with an earlier calculation in this paper shows that this method results in a freezing rate about 10^4 times as great as the method generally practised at present.

B. *Sublimation time.* — An advantage of a method of freeze-drying in which the specimen material is spread in a thin layer is the resultant brevity of the sublimation period. If reasonable assumptions are made as to the efficiency with which the sublimed vapor is carried away from the specimen droplet, a sample calculation can be made of the time taken to sublime an ice layer some 3 μ thick at a temperature of —50° C. Malmstrom (5) has recently published some equations which allow calculations of this nature to be made expeditiously. If the assumption is made that the efficiency of vapor removal is 10 per cent, then a 3 μ layer of ice will sublime at —50° C in about 30 sec. A period of 15 minutes is customarily taken, and this should be ample to effect complete sublimation.

C. *Volatility of suspending media.* — If a suspending medium containing a non-volatile electrolyte is used, the results are disastrous, since a biological suspension in some common electrolyte, such as a phosphate buffer, generally contains far more salt than biological material. Completely volatile suspending media must be employed for those preparations which cannot be suspended in distilled water. Ammonium acetate has been successfully used in the standard spray-drop technique, but cannot be used for freeze-drying, since

the melting point of its eutectic concentration is below —70° C. The requirements placed upon a satisfactory solution for freeze-drying are 1) a melting point above, say, —30° C, 2) complete volatility under conditions of vacuum sublimation, 3) isotonic properties rendering the electrolyte tolerable for biological objects. A preliminary search[1] has indicated that 2 per cent solutions of ammonium succinate or ammonium benzoate, at pH 7.0, may be satisfactory suspending media. Their melting points, in saturated solutions, are about —10° C, and they volatilize apparently completely when the temperature of the specimen is raised to 50° C toward the end of the sublimation period.

D. *Electron microscopy.* — As Anderson has pointed out, frozen-dried specimens of large size are extremely liable to damage by an intense electron beam. This is probably due to the lack of thermal contact between the specimen object and the collodion substrate film. It is found that micrographs of specimens as large as bacteria must be secured at low magnification with an electron beam intensity barely sufficient for focussing. Fortunately, with shadowed specimens, any gross structural change occurring in the microscope can be detected through a lack of perfect fit between object and shadow.

SUMMARY

A method has been developed for extremely rapid freezing, and for fairly rapid vacuum sublimation, of specimens to be examined by electron microscopy. A critical evaluation of the currently practised method of freeze-drying shows that it is inadequate in both its freezing and sublimation aspects. The newly developed method reported here provides for impinging high-velocity micro-droplets of suspension upon a collodion-coated copper surface at a temperature from —75° C to —170° C, and for subliming the resulting thin layers of ice at about —50° C. The time taken for such droplets to pass through the freezing point is estimated at about 10^{-5} seconds. The entire time necessary for a freeze-drying cycle, from spraying to observation in the electron microscope is about 40 minutes. Satisfactory volatile electrolytes for suspending and freeze-drying biological objects appear to be ammonium succinate and ammonium benzoate. Micrographs are shown which indicate that the new method preserves three-dimensional structure, and that negligible surface-tension forces act upon the specimen during sublimation.

[1] These compounds were suggested and tried out by Dean Fraser of this Laboratory.

The work reported here has been aided greatly by the resourceful assistance of Mrs. Laura Tietz.

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ÉTUDE MICROCINÉMATOGRAPHIQUE ET MICROÉLECTRONIQUE DU CENTRE CELLULAIRE DES LEUCOCYTES DES VERTÈBRÉS

A. POLICARD et M. BESSIS

Laboratoire de Physiologie, École Normale Supérieure et Centre National de Transfusion Sanguine, Paris, France

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La présence d'un centre cellulaire dans les leucocytes en dehors des mitoses est, on le sait, connue depuis longtemps (6, 7, 8, 9, 11). Il a été étudié jusqu'à présent à l'aide des méthodes cytologiques classiques, c'est-à-dire dans des conditions qui sont loin d'exclure la formation d'artéfacts. Ainsi s'explique l'imprécision des connaissances à son sujet. En particulier, en dehors des travaux bien connus de Chambers (5) sur le centrosome des œufs en voie de division, les études de cet organe cellulaire sur des éléments vivants en dehors des mitoses sont très rares. En ce qui concerne les leucocytes nous n'en avons relevé que deux (4, 10).

MATÉRIEL ET TECHNIQUES

Nous avons examiné des leucocytes de triton, d'axolotl, de grenouille et des leucocytes provenant de sangs humains. Les préparations ont été obtenues en général en déposant une goutte de sang entre lame et lamelle; pour le sang humain, l'observation a été faite sur une goutte de sang enrichie en leucocytes par centrifugation; les anticoagulants utilisés (citrate de soude, héparine) ne perturbent pas les phénomènes étudiés dans cette note.

Les polynucléaires et les monocytes ont été examinés en outre, après étalement spontané sur film de matière plastique, suivant une technique déjà décrite (1). Dans ces conditions, les leucocytes sont adhérents au support, leur épaisseur est très réduite et, après fixation osmique, le microscope électronique en donne de bonnes images directes (2, 3). Pour cette étude cependant, nous avons préféré utiliser la méthode des pseudo-répliques (12) qui permet d'apprécier le relief cellulaire et montre des détails jusque dans la zone périnucléaire, plus épaisse et habituellement opaque aux rayons électroniques, zone où se trouve habituellement le centrosome. Les phénomènes étudiés se constatent aisément à l'observation microscopique simple, mais, étant donné la lenteur de certains mouvements, la microcinématographie accélérée en permet une bien meilleure étude. Suivant les cas, l'accélération a varié de 10 à 40 fois.

ASPECT EN CONTRASTE DE PHASE

Auprès du noyau, mais cependant nettement séparée de lui, se trouve une masse cytoplasmique homogène, claire, généralement sphérique ou ovalaire, de 3 microns environ. Ses limites sont floues. Elles sont marquées par l'arrêt des granulations: mitochondries des lymphocytes et monocytes et granulations spécifiques des polynucléaires.

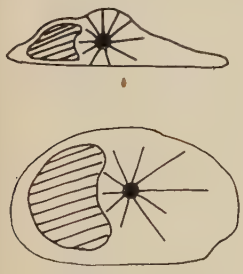

Fig. 1.

Fig. 2.

Fig. 1. Centrosome et zone astérienne vus en coupe (en haut) et en plan (en bas).
Fig. 2. Modifications agoniques dans un lymphocyte. Le noyau devient sphérique, le dessin chromatinien s'estompe, les chondriocontes se transforment en mitochondries, puis envahissent la zone du centre cellulaire (d'après une séquence filmée).

A partir des bords de cette masse cytoplasmique claire et homogène, on constate l'existence de courants radiaires dans le granuloplasma. Les granulations sont entraînées par des mouvements allant de cette masse à la périphérie du granuloplasma et vice versa. Les courants démontrent la nature centrosomique de cette masse claire.

Les déplacements de granulations à partir du centre cellulaire ne sont pas réguliers. Ils présentent des variations et des irrégularités, des sortes d'à-coups assez caractéristiques. Tout se passe comme si les granulations étaient, par moment, entraînées brusquement par la rétraction rapide d'un filament tendu revenant rapidement à sa position primitive. Le fait de ces déplacements et de leur caractère spasmodique n'est pas douteux. Par contre le mécanisme du phénomène demeure indéterminé. On peut se demander s'ils ne sont pas liés à des contractions des fibrilles submicroscopiques du cytoplasma. Notons que, arrivés au centre ou près du centre, les granules retournent en arrière sans jamais franchir la zone claire centrale. Y a-t-il des rapports entre ces déplacements radiaires des granulations et une cyclose générale dans la cellule? Ce point n'a pas pu être précisé.

Très généralement, le centre cellulaire est au contact du noyau. Celui-ci se moule sur lui en se déformant sensiblement. L'aspect réniforme habituel

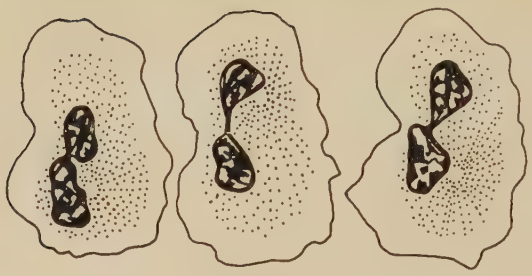

Fig. 3.

Fig. 4.

Fig. 3. Aspect du centrosome et des granulations neutrophiles dans un polynucléaire spontanément étalé. Les figures ont été dessinées à 30 secondes d'intervalle (d'après une séquence filmée).
Fig. 4. Amplitude du mouvement du centrosome (polynucléaire étalé).

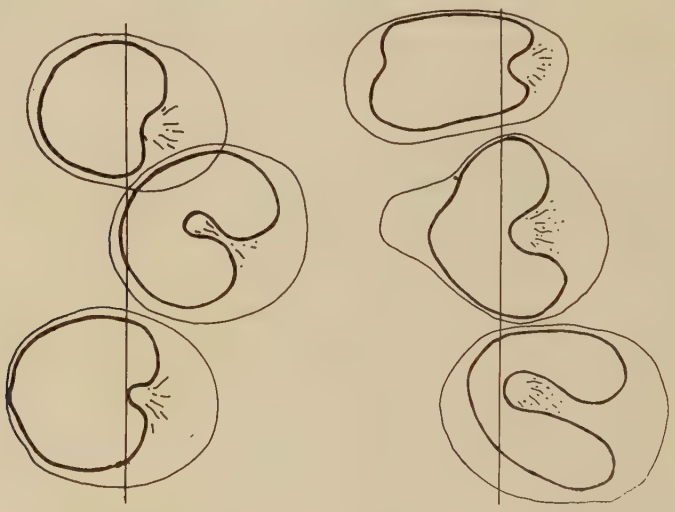

Fig. 5. Oscillations d'un lymphocyte, montrant l'inertie du centrosome qui déforme le noyau (d'après une séquence filmée) (les figures ont été décalées en hauteur).

du noyau des monocytes est précisément dû à la dépression du noyau par le centre. La microcinématographie en accéléré montre ces faits d'une façon indiscutable. Sur les films, le noyau apparaît toujours comme l'élément peu consistant, malléable, se déformant constamment. Par contre, le centre

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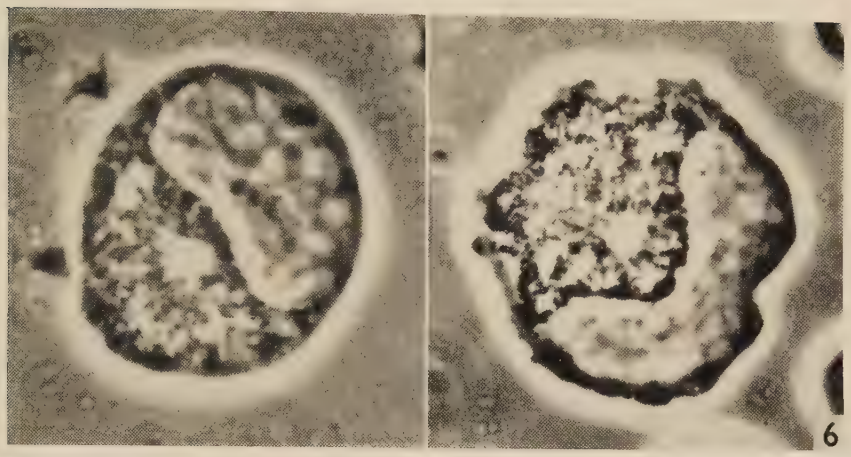
Fig. 6. *Leucocytes du sang humain examinés en contraste de phase.* Deux leucocytes sphériques, montrant la zone centrosomique, délimitée par l'arrêt des granulations neutrophiles.
Fig. 7. *Leucocytes du sang humain examinés au microscope électronique.* Polynucléaire neutrophile spontanément étalé sur »Formvar» — pseudo-réplique au microscope électronique. — Remarquer le relief du centrosome.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cellulaire se montre comme l'élément plus dur, beaucoup plus résistant mécaniquement.

La consistance du centrosome est liée à l'activité vitale de la cellule. Quand on suit les transformations agoniques de l'élément, après la vacuolisation des mitochondries et avant l'homogénisation du noyau, on assiste à une disparition du centrosome par une sorte de liquéfaction. Les granulations cytoplasmiques pénètrent dans le territoire centrosomique. La déformation nucléaire s'éffface. Tout indique que le territoire plus rigide constitué par le centrosome a disparu.

MOUVEMENTS RYTHMIQUES DU CENTROSOME

La microcinématographie met aussi en évidence une mobilité propre du centrosome dans la cellule. Il offre des mouvements oscillatoires indiscutables et tout à fait curieux. Ces mouvements de va et vient ont une amplitude de l'ordre de 3 à 7 microns. Dans les conditions de la prise du film, c'est-à-dire à la température de la chambre, ils ont lieu au rythme moyen d'une oscillation par minute environ. Ils sont donc peu visibles à l'observation microscopique ordinaire, mais ils deviennent évidents sur un film accéléré de 20 à 40 fois. Les oscillations du centrosome ont lieu parallèlement au grand axe du noyau et au plan d'adhérence de la cellule au support.

A la fin de chaque demi-période, il existe un temps de repos de quelques secondes. L'amplitude de ce mouvement varie avec la forme cellulaire et n'est nullement génée par l'étranglement du corps de la cellule. Elle diminue à mesure que la température s'abaisse de 37° à 4° où les mouvements s'arrêtent.

Sur des cellules non fixées au support, les films en accéléré permettent une autre constatation. On voit assez souvent la cellule toute entière osciller autour du centre cellulaire. Tout se passe comme si celui-ci présentait une inertie plus grande que le reste de la cellule. Il constitue une sorte de point fixe autour duquel la cellule décrit des mouvements variés. Le mécanisme de ce processus n'a pas encore pu être déterminé. Peut-être est-il en rapport avec une viscosité plus grande de ses zones périphériques, ce qui le ferait adhérer légèrement au support. Ceci n'est encore qu'une hypothèse non vérifiée.

ASPECT EN MICROSCOPIE ÉLECTRONIQUE

La microscopie électronique sur des leucocytes spontanément étalés et fixés à l'acide osmique, permet diverses constatations se superposant à celles fournies sur le vivant par la microcinématographie en contraste de phase.

Sur des pseudo-répliques ombrées, le centre cellulaire apparaît comme une sorte de colline dominant le plateau constitué par le noyau cellulaire moins élevé, et la couche encore plus basse du cytoplasma. On peut appliquer à ces faits les considérations suivantes:

Dans un élément cellulaire désséché (obligatoirement en raison de la technique utilisée), la hauteur d'une structure dépend des quantités relatives d'eau et de matériaux protéiques (matière sèche) qu'elle comporte. Le centre cellulaire est plus haut que le noyau ou le cytoplasma, vraisemblablement parce que sa teneur en eau est plus faible et sa teneur en substances sèches plus grande. On peut aussi donner de ce fait une autre explication, en supposant que le centre cellulaire aurait une structure réticulaire, avec des fibrilles qui, après fixation ne s'aplatiraient pas. Elles resteraient dressées, les espaces interfibrillaires demeurant vides. Cette hypothèse nous paraît peu vraisemblable. Il n'est cependant pas possible de la rejeter complètement.

RÉSUMÉ

Le centrosome des leucocytes en dehors des mitoses est facilement visible en contraste de phase. Il semble être de densité beaucoup plus grande que le noyau, qui se moule sur lui, et que le reste du cytoplasme non soumis à son influence. Il présente un mouvement oscillatoire tout à fait particulier, dont l'amplitude varie avec la température.

Sur des granulocytes spontanément étalés et examinés au microscope électronique, la zone centrosomique apparaît nettement surélevée par rapport au reste de la cellule.

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STUDIES ON THE CYCLOPHORASE SYSTEM

XXVI. THE LACTIC OXIDASE[1]

H. R. MAHLER, A. TOMISEK[2] and F. M. HUENNEKENS[3]

Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin

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Previous studies from this laboratory on the oxidative pathways of propionate (18, 19) and glucose (22) have shown that the cyclophorase-mitochondrial system of rabbit kidney and liver oxidizes only the D-isomer (22) of lactic acid. When the particulate fraction is supplemented with a soluble α-hydroxy acid racemase found in the supernatant after centrifugation of the original homogenate, it is able to metabolize both isomers with equal facility (23).

D-specificity is unusual for lactic enzymes except for certain bacterial systems (6, 8, 23, 24) which produce or metabolize D-lactate. The lactic enzymes from other micro-organisms (3, 13) plants (7), and animal tissues (2, 5, 13, 26, 31) are reported to be L-specific. It is of interest, therefore, to document the properties of the cyclophorase enzyme and to contrast its properties with those of certain other lactic enzymes which may be derived from animal tissues.

RESULTS

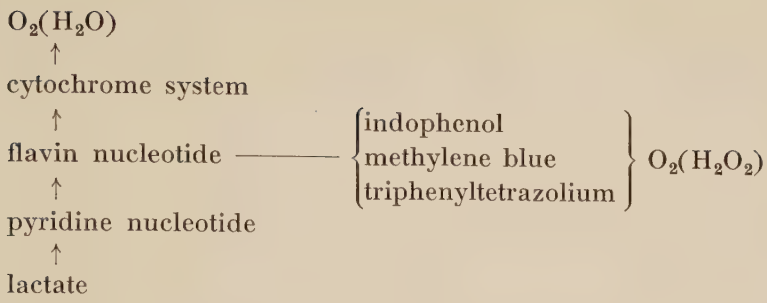
It has been shown previously that many of the oxidative enzymes of the cyclophorase complex exist in the conjugated state (15, 16) in which apoenzyme and coenzyme are firmly linked. Thus, the enzymes function optimally in the absence of added cofactors or coenzymes. The D-lactic oxidase, likewise, conforms to this pattern.

In order to demonstrate the properties of this enzyme, it is necessary to use test systems which arbitrarily assume a given electron transfer scheme. The scheme illustrated below, in which the arrows indicate the direction of electron transfer, has been invoked for all lactic enzymes previously isolated from animal tissues, and also appears adequate for this enzyme as subsequent data will demonstrate.

[1] A preliminary account of this research has been presented at the 121st National Meeting of the American Chemical Society, Milwaukee, Wisconsin, March 30, 1952.

[2] Present Address: Southern Research Institute, Birmingham, Alabama.

[3] Present Address: Department of Biochemistry, University of Washington, Seattle, Washington.



Four possible test systems are suggested by this scheme[1]: (a) the reduction of oxygen (to water); (b) the reduction of oxygen (to hydrogen peroxide) mediated by the interposition of an autoxidizable dye, such as methylene blue or pyocyanine; (c) the anaerobic reduction of various dyes such as methylene blue, indophenol, or triphenyltetrazolium; and (d) the reduction of pyridine nucleotide. The last of these methods, usually carried out spectrophotometrically, is not readily applicable to a particulate system. The other three test systems, however, have been utilized to demonstrate that the cyclophorase enzyme A is specific for the D-isomer, and is conjugated with respect to diphosphopyridine nucleotide (DPN) or else does not use this coenzyme[2] (Table I). It should be noted that similar preparations from kidney, R_3K, give identical results. Other properties of enzyme A are illustrated in Table II, the data for which was obtained in the anaerobic indophenol test system. Although this method suffers from the disadvantage of relatively high blanks (*ca.* 10 per cent of the experimental) when applied to particulate preparations, it has been employed, nevertheless, to facilitate comparison of the particulate lactic enzyme with the various derived enzymes for which the method is more applicable. The data of Table II demonstrate that the cyclophorase lactic enzyme is D-specific, does not require added DPN, is not inhibited by pyruvate (even when added in gross amounts), and is not accelerated by the addition of cyanide. The activity is linearly dependent upon enzyme concentration, the pH optimum is in a broad range between 6.8 and 7.8, and, as shown in Fig. 1, the enzyme is saturated at low substrate concentrations. The last two properties are characteristic of other oxidative enzymes in the cyclophorase complex.

[1] For a description of the symbols pertaining to the lactic acid isomers, see *Arch. Biochem.* **30**, 66, 77 (1951).

[2] Triphosphopyridine nucleotide (TPN) is likewise without effect. Several years ago Haugaard observed that rat liver homogenates did not require DPN for lactic dehydrogenase activity (*J. Biol. Chem.*, **164**, 265 (1946)).

TABLE I

Properties of the Cyclophorase Lactic Oxidase in Various Test Systems.

Additions	A Oxygen uptake (μ moles)	B Oxygen uptake (μ atoms)	C Velocity ($10^3 \times sec^{-1}$)
D-lactate	29.0	5.8	13.1
D-lactate + DPN	25.0	5.2	10.3
L-lactate	−0.9	0.8	1.9
L-lactate + DPN	1.2	1.0	0.7

Experiment A: Each manometer cup contained 1.0 ml of R_2L, 5 μ moles citrate, 20 μ moles phosphate buffer pH 7.3, 3 μ moles 5-adenylic acid, 4 μ moles $MgCl_2$, 10 μ moles of the lactate isomer (where indicated) and 1000 μgm of DPN (where indicated). NaOH in the center well, total liquid volume 3.0 ml. Manometric procedure: oxygen in the gas space, temperature 38°, five minute thermal equilibration prior to closing taps, total duration of experiment 90 minutes, oxygen uptake values corrected for blank in the absence of substrate.

Experiment B: Each manometer cup contained 1.0 ml R_2L, other components similar to those described under (a), with the following additions: 100 μ moles KCN (neutralized to pH 7.3), 200 μgm pyocyanine, and the omission of citrate. Experimental conditions similar to those of experiment A.

Experiment C: Each Thunberg tube contained in the main compartment: 1.0 ml R_2L (diluted 1 : 1 with 0.9 per cent KCl), 30 μ moles phosphate buffer pH 7.3, 100 μ moles KCN (neutralized to pH 7.3), one drop capryl alcohol, 50 μ moles of lactate isomer and 250 μgm of DPN where indicated. 500 μgm 2,6-dichlorophenolindophenol was placed in the side arm. Total liquid volume 3.0 ml. Tubes evacuated for five minutes, equilibrated at 38° for two minutes, experiment started by tipping and mixing. Velocity is expressed as the reciprocal of the reduction time; velocities are corrected for blanks in the absence of substrate.

TABLE II

Further Properties of Cyclophorase Lactic Oxidase.

Additions	Velocity ($10^2 \times sec^{-1}$)
Complete system	3.0
With 50 μ moles L-lactate	0.2
With 50 μ moles D-lactate	3.4
DPN omitted	3.5
Cyanide omitted	3.5
10 μ moles pyruvate	3.5
100 μ moles pyruvate	3.2

The complete test system, placed in the main compartment of Thunberg tubes consisted of 1.5 ml R_2^1L (diluted 1 : 2 with 0.9 per cent KCl), 50 μ moles phosphate buffer pH 7.3, one drop capryl alcohol, 250 μgm. DPN, 100 μ moles KCN and 100 μ moles D,L-lactate (except where the isomers were substituted). 500 μgm 2,6-dichlorophenolindophenol in the side arm. Total liquid volume 3.0 ml. Other conditions as in experiment C, Table I.

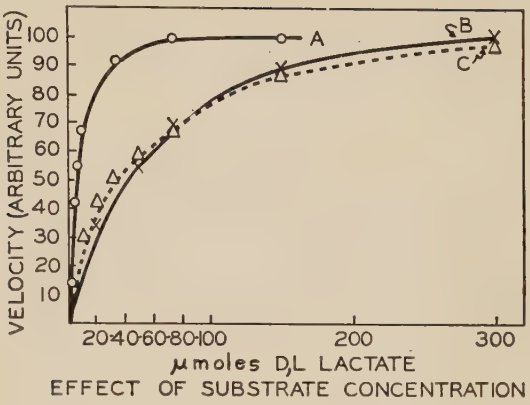


Fig. 1. Test systems used were those of Table II for enzyme A, Table IV for enzyme B, and Table V for enzyme C. D,L-lactate was added in the amounts shown.

Since this enzyme displays an unexpected optical specificity, it seemed necessary to establish the identity of the oxidation product. In the presence of malonate which blocks the citric acid cycle, pyruvate accumulation can be demonstrated, as shown in Experiment A of Table III. Pyruvate was estimated by the modified method of Friedemann and Haugen (17) which does not estimate acetoacetate. In addition, the 2,4-dinitrophenylhydrazone of pyruvate was isolated and identified by a specific chromatographic method (*cf.* Methods). Only a negligible amount of acetoacetate could be found when tested for by the aniline-citrate method. This negative result was surprising in view of the fact that liver particles contain the system capable of converting pyruvate to acetoacetate (28):

$$2\ CH_3COCO_2H + O_2 \rightarrow CH_3COCH_2COOH + 2\ CO_2 + H_2O.$$

Under similar experimental conditions pyruvate was only partially converted to acetoacetate (Experiment B, Table III). At these concentrations, malonate inhibits not only the citric acid cycle, but also the system converting pyruvate to acetoacetate. Pyruvate in massive quantities partially overcomes this latter inhibition; in smaller quantities, however, as in its generation from lactate, it is incapable of doing so.

Other devices have been used to show pyruvate accumulation: (a) trapping with cyanide (Experiment C, Table III); and (b) by using kidney cyclophorase, which does not catalyze the conversion of pyruvate to acetoacetate (12), and the omission of "sparker", so as to permit the accumulation of pyruvate (Experiment D, Table III).

It is evident from the above data that the cyclophorase lactic oxidase

TABLE III

Pyruvate Accumulation During Oxidation of D-Lactate.

Experiment	A	B	C	D
Substrate	D-lactate	Pyruvate	D-lactate	D-lactate
Preparation	R_2L	R_2L	R_2L	R_4K
"Block"	Malonate	Malonate	CN^-	No sparker
Oxygen uptake (μ moles)	16.3	23.0	1.75	2.10
Change in pyruvate (μ moles)	+4.9	−28.0	+3.4	+4.0
Acetoacetate (μ moles)	≤1.0	12.3	0	0

Equations: lactate $+\frac{1}{2}O_2 =$ pyruvate $+ H_2O$.
pyruvate $+\frac{1}{2}O_2 = \frac{1}{2}$ acetoacetate $+ CO_2$.

Experiment A: Each manometer cup contained 1.0 ml R_2L, 5 μ moles citrate, 30 μ moles D, L-lactate, 10 μ moles malonate, all other components and conditions as in experiment A, Table I.

Experiment B: Similar to experiment A, except 31.2 μ moles of pyruvate were substituted for the lactate + citrate.

Experiment C: Each manometer cup contained 1.0 ml R_2L, 400 μgm pyocyanine, 100 μ moles sodium cyanide, 20 μ moles D-lactate and all other components as before.

Experiment D: Each manometer cup contained 1.0 ml R_4 K, 20 μ moles D-lactate, all other conditions and components as above.

TABLE IV

Properties of Enzyme B.

Additions	Velocity ($10^3 \times sec^{-1}$)
Complete system	24.0
With 100 μ moles L-lactate	25.2
With 100 μ moles D-lactate	0
DPN omitted	0
Cyanide omitted	10.2
10 μ moles pyruvate	14.4
100 μ moles pyruvate	0.4

The complete test system consisted of 0.3 ml enzyme, 50 μ moles of phosphate buffer pH 7.3, 225 μ moles D,L-lactate (except where the isomers were substituted), 100 μ moles KCN, 250 μgm DPN, and all other components and conditions as in Table II.

exhibits several properties not shared by other lactic oxidases derived from animal tissues, including kidney and liver. Vestling and Knoepfelmacher (35) have described a lactic enzyme from rat liver, which, from its method of preparation and source, would appear to be related to the cyclophorase counterpart; its properties, however, are quite different.

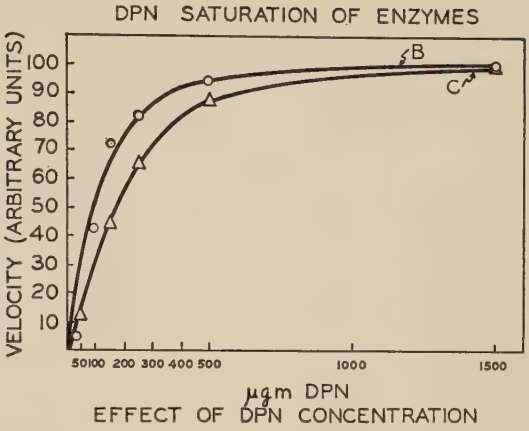


Fig. 2. Test systems used were those of Table IV for enzyme B and Table V for enzyme C. DPN = diphosphopyridine nucleotide, added in the amounts shown.

Although this type of lactic enzyme, called enzyme B, is customarily prepared by homogenizing rat liver in distilled water with a Potter-Elvehjem homogenizer, a similar preparation can be obtained by prolonged homogenizing of rabbit liver in dilute saline with the Waring blendor. Furthermore, a cyclophorase preparation of washed liver particles (R_3L) can be changed to a type B preparation by further homogenization in the presence of the original supernatant (S_1L), or, less effectively, in the presence of deionized water. Full detail of these preparations are given in the Experimental Section.

The anaerobic reduction of dyes offers a convenient means of testing this enzyme. In Table IV, the indophenol test is employed to demonstrate certain properties of enzyme B. It is seen that the enzyme is L-specific, does not function in the absence of DPN, is partially inhibited by pyruvate, and is accelerated by the addition of cyanide. The saturation with respect to substrate is shown in Curve B of Fig. 1, while the saturation with respect to the primary prosthetic group, DPN, is illustrated as Curve B in Fig. 2. The pH optimum under these test conditions lies between 8.5 and 8.9.

A comparison of enzyme B with the original cyclophorase lactic oxidase suggests the relation previously established for other conjugated pyridinoproteins and their dissociated counterparts (16, 17, 34); this point will be considered further in the Discussion Section. In further contrast, certain other lactic enzymes from animal tissues which have been isolated in a soluble form (13, 26, 31), differ in properties from both enzymes A and B. It seemed of interest, therefore, to prepare a soluble lactic enzyme directly from the cyclophorase preparation. Various methods known to cause dissociation of coenzymes and possible solubilization of the apoenzymes were

TABLE V

Properties of Enzyme C.

Additions	Velocity ($10^3 \times sec^{-1}$)
Complete system	7.2
With 100 μ moles L-lactate	7.0
With 100 μ moles D-lactate	0
DPN omitted	0
Cyanide omitted	4.3
10 μ moles pyruvate	2.9
100 μ moles pyruvate	0.2

The complete test system consisted of 0.5 ml enzyme, 50 μ moles phosphate buffer pH 7.3, 200 μ moles D,L-lactate (except where the isomers were substituted), 100 μ moles KCN, 250 μgm DPN, and all other components and conditions as in Table II.

explored in this case. Such procedures as: acetone precipitation, aging at low temperatures (*ca.* 4°) in the presence and absence of capryl alcohol, repeated freezing and thawing, and ammonium sulfate fractionation, each achieved some measure of success, but with large concomitant losses of activity. On the other hand, an equally simple procedure, prolonged exposure of the cyclophorase preparation to large volumes of cold saline solution, was found to achieve both solubilization and good recovery of activity. Substitution of deionized water for the saline led also to solubilization, but with a diminished recovery of activity. As previously shown (16, 17), solubilization of oxidases varies as a function of time with each individual cyclophorase preparation; in all cases, however, the bulk of the total extractable activity had been solubilized at the end of twenty-four hours. This preparation, enzyme C, is a truly soluble lactic oxidase since the activity resides entirely in the supernatant fraction after high speed centrifugation to remove microsomal particles. Further discussion of the solubilization process will be deferred to a later section of this paper. Reduction of DPN, followed spectrophotometrically, is a satisfactory assay method for the soluble enzyme C. For comparative purposes, however, the properties of this enzyme have been documented with the aid of the indophenol test system. Using this test system for enzyme C, the blanks without substrate were small in contrast to the high blanks for enzymes A and B in this test system.

Enzyme C is L-specific, requires DPN, and is inhibited by its product,

TABLE VI

Distribution of Activities between Fractions.

Enzyme	Fraction	Velocities ($10^3 \times sec^{-1}$)			
		Without DPN		With DPN	
		D-Lactate	L-Lactate	D-Lactate	L-Lactate
A	Residue	9.0	0.3	8.0	0.9
C	Residue	2.0	0.2	1.2	0.2
	Supernatant	0.0	0.0	0.2	12.6

Enzyme A was prepared by mixing a R_2L preparation with an equal volume of 0.9 per cent KCl.

Enzyme C was prepared by storing enzyme A for 36 hours at 4° C. and then separating the residue and supernatant fractions by centrifugation for 30 min. at 20000 × g. The residue was diluted to the original volume with fresh saline.

The test system was essentially that described in Table II with the following components: 1.0 ml enzyme, 50 μ moles phosphate buffer pH 7.3, 100 μ moles KCN, one drop capryl alcohol, 250 μgm DPN, 100 μ moles lactate isomers, and 500 μgm 2,6-dichlorophenol indophenol in the side arm.

TABLE VII

Variation of Activities with Time of Incubation.

Incubation Time hrs.	Total Units A	Total Units C
0	846	0.0
2		111
4		225
24		452
46	80	573
94		655
192	0.0	711

Experiment identical with that described in Table VI, except that aliquots were withdrawn at the times shown. Measurements for the two types of activities performed as described previously. Total units = (velocity/volume of enzyme per test) × total volume enzyme solution.

pyruvate, unless the latter is trapped by cyanide (Table V). Since the addition of a soluble diaphorase (11) does not enhance the activity in this test system, it would appear that the soluble enzyme preparation also contained diaphorase. The activity is linear with respect to the concentrations of both enzyme and dye. The pH optimum of this enzyme is more sharply defined

than it is for enzymes A or B, but the position of the optimum varies between 7.5 and 8.5, depending upon the exact conditions of the preparation. The effect of lactate concentration is shown in Curve C, of Fig. 1, while the saturation with respect to DPN is illustrated as Curve C, in Fig. 2.

Since the soluble enzyme C has been derived from liver cyclophorase, *i.e.* enzyme A, the question arises whether the appearance of enzyme C is paralleled by a corresponding disappearance of enzyme A. In a large-scale preparation of enzyme C, aliquots were withdrawn at various time intervals and separated by centrifugation into the supernatant and particulate fractions. Each fraction was tested for activity towards the two enantiomorphs of lactic acid and the requirement for added DPN. As shown in Table VI, enzyme A is found only in the particulate fraction, while enzyme C is localized entirely in the supernatant. Furthermore, in line with the results of Table VII, when the activities are expressed in approximately the same units, the decline in enzyme A is accompanied by a corresponding increase in enzyme C so that the total activity remains essentially constant.

DISCUSSION

As an aid in the discussion, the salient properties of the three lactic enzymes described in this paper are compared in Table VIII. It will be noted that the cyclophorase enzyme A exhibits two properties not shown by enzymes B and C, nor by other lactic enzymes derived from animal tissues, *viz.* specificity for the D-isomer and no requirement for added DPN. These properties *per se* are not entirely unique; D-specificity[1] is shown by certain bacterial lactic enzymes (8), while L-specific plant (7) and yeast lactic oxidases (3, 13) not requiring DPN, have also been reported. The soluble plant enzyme appears to be completely conjugated, but the nature of its prosthetic group, or groups, is as yet unkown. The yeast enzyme is believed to operate directly through cytochrome b_2 without intervention of pyridine or flavin nucleotides (1). The question naturally arises whether the cyclophorase enzyme might also utilize some prosthetic group other than pyridine nucleotide. The customary procedures for splitting out bound pyridine nucleotide and then reactivating the apoenzyme with added pyridine nucleotide, have been employed to investigate this point. These procedures do, in fact, lead to apoenzymes such as enzyme B, still on the particles, or enzyme C in solution —

[1] Km values are calculated by the Lineweaver-Burk method (*J. Am. Chem. Soc.*, **56**, 658 (1934)) from the data of Figs. 1 and 2 and are in units of moles/liter. In the case of substrate, the values are with respect to the isomer utilized.

TABLE VIII

Summary of Properties of Lactic Oxidases.

Property	A	B	C
DPN requirement	—	+	+
Substrate specificity	D	L	L
Product inhibition	—	+	+
K_m for substrate[1]	1.3×10^{-3}	13.7×10^{-3}	12.0×10^{-3}
K_m for DPN[1]	—	5.0×10^{-4}	8.6×10^{-4}
pH Optimum	7.0—8.0	8.0—9.0	7.5—8.5

which do not function unless supplemented with DPN. Since these derived enzymes, however, show opposite substrate specificity from their supposed precursor, their exact relation to enzyme A is still in doubt. No procedure has yet been found which will render the cyclophorase enzyme DPN-requiring while maintaining its D-specificity.

Some mention should also be made of certain features of enzymes B and C. An inspection of Table VIII reveals that the properties of both enzymes B and C closely resemble those of other dissociated lactic enzymes from animal tissues (13, 26, 31) The difference in pH optima between enzymes B and C is not considered to be significant since variations in the pH optimum of either enzyme can be produced by slight alterations in the conditions of preparation, and even by prolonged aging of the enzyme at 4°. Furthermore, in the usual indophenol test system where the primary dehydrogenase is coupled to the dye through a diaphorase, the pH optimum is not necessarily a property of the first step.

A comparison has been made recently between the cyclophorase malic oxidase and its dissociated counterpart (15). The cyclophorase enzyme does not require added DPN, is not inhibited by oxalacetate and has a $K_m = 2 \times 10^{-3}$ M with respect to its substrate. On the other hand, the dissociated enzyme — either still on the particles or in solution — requires DPN, is inhibited by oxalacetate and has a $K_m = 25 \times 10^{-3}$ M with respect to its substrate.

The observations made in this paper and in previous communications

[1] Several investigators (6, 13, 23, 24) have observed that *unbroken cells* of certain microorganisms metabolize either isomer of lactic acid with equal facility, but that upon isolation an L-specific enzyme is obtained (24). In an analogous manner, rabbit liver and kidney cyclophorase, supplemented with the supernatant fraction (racemase), can oxidize either isomer, but the particles alone contain a D-specific oxidase (18). Particulate preparations from pigeon breast muscle and baker's yeast are L-specific but in the presence of the above racemase both isomers are oxidized (19). We have not yet investigated whether the yeast and pigeon breast supernatant fractions likewise contain the racemizing enzyme.

(18, 19) may be summarized as follows: 1) In mitochondrial preparations, (R_3K or R_3L), only D-lactate is utilized; 2) when supplemented by a partially purified soluble fraction from the first supernatant (S_1K or S_1L), the mitochondrial preparation will utilize either isomer at equal rates — this effect has been attributed to the presence of a soluble α-hydroxy acid racemase, and has been shown to be dependent upon oxidative phosphorylation; 3) a well-washed mitochondrial preparation (R_3K or R_3L), unable to oxidise L-lactate, can be extracted with dilute saline solution to yield a soluble, L-specific lactic oxidase. Concomitantly with the appearance of the L-specific enzyme in the extract, the D-specific enzyme disappears from the particles.

It is difficult to conceive of an adequate explanation for the facts of item 3) above. One possible explanation might be that both the D- and L-specific enzymes are present in the original mitochondrial preparation, but that the L-activity is masked. Upon solubilization the L-enzyme is liberated while the D-enzyme is destroyed. Thus, if two separate enzymes are involved, the alteration in properties is not unexpected. The existence in a cell of two separate enzymes catalyzing the same reaction has been given recent support by the work of Kornberg and Pricer (25) who isolated both a DPN and a TPN requiring isocitric dehydrogenase from baker's yeast. The liberation of an enzymatic activity, on destruction of mitochondria, previously only latent or masked in the particles, has recently been reported by Berthet and DeDuve (4).

In the analogous cases of the malic oxidase from rabbit liver and kidney and the lactic oxidase from pigeon breast muscle, the same pattern of alteration in properties accompanies the removal of bound DPN, with the exception that the optical specificity with respect to substrate remains unaltered, *i.e.* whether the enzymes are conjugated or DPN-requiring, they are specific for L-malate and L-lactate, respectively (21).

EXPERIMENTAL

Enzyme Preparations

Enzyme A (*mitochondrial*). Mitochondrial preparations used in this investigation followed the description of Sarkar, Beinert, Fuld and Green (33) for rabbit liver; the tissue was homogenized in sucrose, the suspension diluted with isotonic KCl and washed once with KCl. These preparations were used either directly or after dilution with KCl and are referred to as

R_2L. Kidney preparations have also been described previously (14, 18). They were generally taken to the third or fourth residue stage and are then referred to as R_3K or R_4K, respectively.

Enzyme B. Numerous variations, depending on starting material, homogenization medium, type of homogenizer, etc., have been employed. Two modifications will be described briefly:

1. *From rabbit liver.* — Several grams of freshly excised, chilled rabbit liver were homogenized with four volumes of deionized water in a glass Potter-Elvehjem homogenizer for four minutes. Care was taken to keep the temperature close to 0°. The homogenate was centrifuged at 2300 ×g for 5 minutes in the angle head of the refrigerated International centrifuge. The residue was discarded and the pink supernatant used. The latter could be dialyzed, lyophilized, or frozen and stored at —10°. The frozen preparation was found to be stable for several months and did not lose activity even upon repeated freezing and thawing.

In case of larger scale preparations, homogenation could be replaced by blendorization in a Waring blendor at full speed for five minutes. The enzyme was worked up as before and in control experiments showed specific activities comparable to those of enzymes prepared by means of the glass homogenizer.

2. *From R_2L.* — One volume of R_2L was homogenized with an equal volume of deionized water in a glass Potter-Elvehjem homogenizer for four minutes at 0°. After centrifugation as before, a preparation was obtained which was identical in specificity and all other properties with that obtained from fresh liver.

Enzyme C. One of the most efficient and simple methods for the preparation of this enzyme consisted in incubating one volume of a preparation of liver mitochondria after dilution with an equal volume of 0.9 per cent KCl for varying lengths of time at about 1°. The bulk of the extractable activity was obtained after 24 hours, while the particles could be removed by centrifugation at high speed for thirty minutes in the high speed head of the International centrifuge. When frozen, these soluble preparations were stable for periods of one to several weeks.

The dilution with saline could best be effected by means of homogenization. If more than one volume of KCl was used, solubilization of the enzyme could usually be accelerated, but the final yield (after about 3–5 days) was the same as long as not more than four volumes of diluent were employed. It was found expedient to resuspend the particles in the extracting medium by homogenization about every twelve hours.

MATERIALS AND METHODS

Experimental procedure in manometric and Thunberg experiments has already been outlined in the legends of Tables I and II. pH Optima were determined by measuring the velocity of dye reduction as a function of pH in a series of Thunberg tubes, the actual pH in each being measured at the conclusion with a glass electrode.

Determinations

Pyruvate. The method of Friedemann and Haugen (10) was used. Spectrophotometry was carried out by means of a Coleman Universal spectrophotometer and readings were taken at 450 mμ using a standard curve prepared by means of known amounts of pyruvate carried through the procedure.

When the experimental mixture contained a dye such as indophenol or methylene blue, or a carbonyl reagent like cyanide, the method has been modified as follows: An appropriate amount of a 5 per cent trichloracetic acid (TCA) filtrate (containing $\simeq$ 250 μgm pyruvate) was placed into a 25 ml Erlenmeyer flask and enough florisil (Floridin Company, Warren, Pa.) was added with swirling until the supernatant was completely colorless; 25–30 mg was usually sufficient. The mixture was then filtered or centrifuged with thorough washing with 5 per cent TCA, and the supernatant and washings placed in a 25 ml volumetric flask. After making up to volume by means of 5 per cent TCA, a suitable volume (containing approximately 20 μgm pyruvic acid) was used either directly or, if the mixture contained CN^-, it was pipetted into a test tube, chilled in an ice bath and 6 N NaOH was then added until the pH rose above 13. The mixture was poured into 1 ml of phenylhydrazine reagent, prepared as usual, and the subsequent procedure was identical to the published method.

Identification of Pyruvate. Pyruvate was identified either by means of the Friedemann-Haugen analytical determinations or by the following procedure: After the addition of 2,4-dinitrophenylhydrazine, the hydrazones were extracted into ether and this solution passed slowly through a microchromatographic column of Florisil (50–80 mesh). The hydrazones were adsorbed in an orange-yellow band at the top of the column. The chromatogram could be developed with chloroform, followed by butanol which removed the 2,4-dinitrophenylhydrazones of all other carbonyl compounds tested (including all simple aldehydes and ketones, and all other keto acids, such as oxalacetic, oxalsuccinic, α-ketoglutaric, acetoacetic, etc.) except that of pyruvate which was held in a narrow, brilliant, golden band at the top of the column. The compound was finally eluted by means of a butanol-glacial acetic acid (1:1) mixture. When the solution was extracted into base, followed by neutralization, it was found to yield the absorption spectrum typical for the 2,4-dinitrophenylhydrazone of pyruvate (29).

Acetoacetate. Acetoacetate was estimated by the manometric aniline citrate method (31).

Substrates. Samples of lactate isomers suitable for enzymatic work were prepared as follows: a. *L(—) lactic acid* was prepared by repeated recrystallization of zinc ammonium *L(+)-lactate* (Eastman) from 4 M ammonium hydroxide. After this procedure solutions of the salts showed satisfactory optical rotations. They could be converted to the free acid by passage through a cation column (Amberlite 100-H). L(+) Lactate

samples prepared by neutralization of the acid were completely active with muscle lactic dehydrogenase (27). b. *D*(+) *lactic acid* was obtained by resolution of commercial 85 per cent lactic acid (Analytic Reagent Grade) through the morphine salt (20, 30). The optical rotations of four different samples of D(—) zinc lactate gave an $[\alpha]_D = -7.01 \pm 0.15$ (c = 5.07), calculated for the dihydrate, compared to $[\alpha]_D = -7.20 \pm 0.10$ (c = 5.00) as given in the literation (30). Salts were converted to the free acid as described above. When tested with muscle lactic enzyme, this isomer was completely inactive, but it did not depress the activity of the L-isomer even when present in large excess.

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STUDIES ON THE CYCLOPHORASE SYSTEM

XXVII. CYCLOPHORASE ACTIVITY IN A NON-MITOCHONDRIAL SYSTEM OF PIG HEART MUSCLE[1]

D. E. GREEN, H. BEINERT[2], M. FULD, D. GOLDMAN[2], M. H. PAUL[2], and N. K. SARKAR[3]

Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin

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In previous papers of this series the reaction sequences characteristic of the cyclophorase-mitochondrial (CM) system have been dealt with in some detail. These reactions include: a) complete oxidation of members of the citric acid cycle to carbon dioxide and water (7); b) the sparked oxidation of acetate and acetoacetate to carbon dioxide and water (6); c) fatty acid oxidation (2, 6), and d) oxidative phosphorylation (3). The study of these reaction sequences was undertaken as an integral part of the systematic documentation of the properties of the CM system. Other laboratories have made important contributions to our knowledge of these processes notably those of Barron, Breusch, Cohen, Cori, Hogeboom, Hunter, Kalckar, Lardy, Lehninger, Leloir, Leuthardt, Ochoa and Potter.

Since 1947 many unsuccessful attempts have been made in our laboratory to reconstruct the consecutive reactions of the cyclophorase complex of enzymes in non-mitochondrial systems of liver and kidney. The failure of these previous attempts raised the possibility that certain reaction sequences could proceed only within the organization of the mitochondrial unit. The experiments which are reported in this communication establish that the basic reactions of the cyclophorase system can be reproduced qualitatively if not quantitatively in a non-mitochondrial system prepared from pig heart muscle. This demonstration does not necessarily minimize the significance of organization within the mitochondrial unit, but it does point up that previous failures of reconstruction must be laid at the door of enzyme inac-

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[1] A report of this work was given at the Symposium on Phosphorus Metabolism held at Johns Hopkins University in July, 1951.

[2] Postdoctoral Trainee of the Heart Institute.

[3] Williams-Waterman Fellow in Enzyme Chemistry.

tivation rather than loss of essential organization. Considering the large number of constituent enzymes involved in processes such as fatty acid oxidation and the citric acid cycle it is more remarkable that reconstruction could be successful at all rather than that it has met with difficulties. The loss of a single enzyme would have been sufficient to nullify the reconstruction of any one reaction sequence.

METHODS

Preparation of Enzymes

Fresh pig heart muscle obtained from the packing house is cooled in ice and then diced when brought to the laboratory (within 40 minutes). One part of muscle is blendorized with four parts of cold 0.02 M phosphate buffer of pH 7.4 for 3 minutes. The mixture is centrifuged for 20 minutes at 2000 × g — the residue being discarded. The supernatant is acidified to pH 6.0 with hydrochloric acid and the mixture is centrifuged for 10 minutes. The residue is again discarded. The supernatant is now acidified to pH 5.4 and the mixture is centrifuged for 10 minutes. The residue is resuspended in 10 volumes of 0.02 M phosphate buffer of pH 5.4 and recentrifuged. The washed residue is taken up either in 0.02 M phosphate buffer of pH 7.4 or in 0.3 M sucrose — the pH in both cases being adjusted to pH 7.4. For every 100 g of starting material of diced muscle, the residue should be brought to a final volume of 10 ml. The dry weight of the suspension is about 30 mg per ml. The fraction precipitated at pH 5.4 is particulate in nature, corresponding to what is usually referred to as the microsomal fraction.[1] For simplicity of description, this fraction will be referred to henceforth as the "5.4" fraction or enzyme.

[1] It may be appropriate to summarize at this point the evidence that the "5.4" particulate fraction is non-mitochondrial in nature. a) The intact mitochondrial unit contains all the enzymes and cofactors necessary for the whole gamut of the reactions which it catalyzes. The "5.4" fraction catalyzes none of these reactions except the oxidation of succinate to fumarate, unless supplemented with a group of soluble enzymes and coenzymes. b) The mitochondrial unit is destroyed or severely damaged by any of several procedures employed in preparing and storing the "5.4" fraction *e.g.*, alternate freezing and thawing, homogenization in the Waring blendor for three minutes and exposure to 0.02 M phosphate buffer and pH 5.4. c) The sedimentation characteristics of the mitochondria under the conditions of the preparation are such that if any intact mitochondria survived the homogenization in the Waring blendor, they would be present in the first two fractions which are discarded. d) Examination of the "5.4" preparation in the phase microscope, for which we are indebted to Dr. J. Harman, discloses a vast preponderance of submicroscopic units, and a negligible percentage of mitochondrial bodies of irregular size and shape. The possibility has to be considered that these few, residual (damaged) mitochondrial elements are responsible for the activity of the reconstructed system. If these were intact mitochondria, they would show no requirement for soluble enzymes and coenzymes. Furthermore, the Qo_2 of the system would be very small if the activity were referable to a few mitochondria diluted by a large amount of inert protein. Actually the Qo_2 of the reconstructed system (100–200) is of the order of the highest values found with homogeneous suspensions of liver, kidney and heart mitochondria. In summary, there is a good deal of evidence to justify treating the "5.4" fraction as essentially non-mitochondrial in nature, both from a functional and morphological point of view. The "5.4" fraction is usually considered to be microsomal in nature. However, no effort has been made in this study to characterize the particles of the "5.4" fraction other than as non-mitochondrial particles.

All manipulations are carried out at 0° as soon as the hearts are obtained from the packing house. Adequate cooling precautions should be observed particularly when the pH is reduced to 5.4.

The stability of the "5.4" enzyme is markedly affected by the medium in which it is suspended and kept frozen at −10°. Stability is much greater in the sucrose medium. However, the phosphate medium is more suited for solubilization of enzymes intimately associated with the particles of the "5.4" enzyme.

The supernatant fluid, after removing the pH 5.4 precipitate, is neutralized to pH 7.0 and saturated 80 per cent with respect to ammonium sulfate. The precipitate is filtered, press-dried and taken up in the minimum volume of 0.02 M phosphate buffer and then fractionated with ammonium sulfate. The fractions between 25–40 per cent and 40–65 per cent saturation have been found to contain the components of interest to the present investigation. The final volume of each dialyzed fraction is adjusted so that there are 10 ml of solution for every 100 g of starting material of heart muscle. The different soluble fractions will be designated by notations such as S_{50-65}, where the subscripts refer to the limits of ammonium sulfate concentrations within which the fraction was obtained. The dry weight of the S_{50-65} fraction is about 35 mg per ml.

Preparation of the Coenzyme Concentrates. As sources of crude nucleotide coenzymes, pig liver, pig heart, and dried brewer's yeast have been used — the last being the preferred source. The concentrate from pig liver and brewer's yeast was prepared by the method of LePage and Mueller (5), which consists in adsorbing nucleotides from a boiled extract on to charcoal, followed by pyridine elution and acetone precipitation. The crude acetone powder was used by LePage and Mueller as the starting point for the preparation of pure TPN.[1] The concentrate from pig heart was obtained by precipitating nucleotides from a boiled extract with a slight excess of lead acetate, decomposing the lead salt with sulfuric acid (to pH 2) and after removing lead sulfate, precipitating the active material with 5 volumes of acetone. To remove the last traces of lead, the acetone powder was rubbed in water, the insoluble residue removed and the solution then gassed with H_2S and nitrogen successively. The solution was alternately frozen and thawed three times, and the precipitate of PbS removed by high-speed centrifugation. The acetone powders were usually made up to 5 per cent solutions, and these after neutralization and clarification were kept frozen at −10°. There was no loss of activity on storage. From 1–5 mg were needed to saturate the test system with respect to coenzyme. CoA of high purity (50–90 per cent) was prepared from dried brewer's yeast by the method of Beinert *et al.* (1).

RESULTS

Oxidation of Citric Cycle Intermediates. When the "5.4" particulate enzyme fraction is supplemented with the soluble S_{50-65} fraction and the coenzyme concentrate, the resulting system can catalyze the rapid aerobic oxidation

[1] The following abbreviations will be used throughout: TPN (triphosphopyridinenucleotide), DPN (diphosphopyridinenucleotide); AMP (adenosine-5-phosphate); ADP (adenosinediphosphate); ATP (adenosinetriphosphate) and CoA (acetylation coenzyme).

TABLE I

Components Required for Oxidations of Citric Acid Cycle.

	μ atoms oxygen absorbed in 50 min.		
	fumarate	citrate	α-keto-glutarate
Complete system	46	42	45
no "5.4" enzyme	0	1	0
no coenzyme concentrate	1	3	4
no $S_{50\ 65}$	14	6	4
no substrate	6	6	6

The complete system contained 0.4 ml of "5.4" enzyme, 0.5 ml of S_{50-65}, coenzyme concentrate (5 mg), 30 μ moles substrate, cocarboxylase (100 μg), 0.1 ml of 0.02 M $MgCl_2$, DPN (500 μg) and TPN (500 μg). Final vol. 3.0 ml.

TABLE II

Complete Oxidation of Members of Citric Acid Cycle.

Substrate	μ moles	μ atoms oxygen		
		observed	theory	per cent theory
α-ketoglutarate	18	142	144	99
Citrate	15	109	135	81
Succinate	20	159	140	114
Fumarate	22	130	132	98
Oxalacetate	15	0	75	0
Oxalacetate (7.5) + pyruvate (7.5)[1]	15	69	75	92

Each manometer cup contained 0.5 ml of the "5.4" enzyme, 0.5 ml of S_{50-65}, 5 mg of coenzyme concentrate, 0.1 ml of 0.02 M $MgCl_2$, final vol. 3.0 ml, 38°, 3 hr. incubation, oxygen in gas phase.

of any member of the citric acid cycle with a Qo_2 of 100–200, depending upon the substrate. Table I shows the dependence of the overall rate of oxidation of citric cycle intermediaries upon each of the three components. It should be noted that in addition to the coenzyme concentrate, other cofactors are routinely added to the system *viz.* cocarboxylase, magnesium chloride, DPN and TPN. Thus the effect of the concentrate is additional to that of any of these supplements.

In the presence of the three main components of the pig heart system, all the members of the citric acid cycle were oxidized to CO_2 and H_2O (cf. Table II). The agreement between the observed and theoretical oxygen

[1] Supplemented with 8 μ moles ATP.

TABLE III

Effect of Glutathione on the Rate of Oxidation of Citrate.

	Glutathione added per flask (μ moles)	Oxygen absorbed (μ atoms) in minutes					
		20	40	60	80	100	120
A	0	38	81	111	125	131	132
	10	42	95	134	154	163	166
	30	41	93	135	160	172	179
	60	41	95	140	169	187	200
	90	40	97	147	179	202	218
B	0	14	32	61	80	90	92
	90	14	35	64	103	146	179

The complete system contained 0.5 ml of "5.4" enzyme, 1.0 ml of $S_{50\ 65}$, DPN (1000 μg), TPN (400 μg), cocarboxylase (200 μg), 2 μ moles of magnesium chloride, crude liver coenzyme concentrate (2.5 mg) and 30 μ moles of citrate in a final vol. of 3.0 ml. The oxygen uptake is corrected for the corresponding blank without citrate.

uptake values for complete oxidation is satisfactory considering some of the experimental difficulties such as the uncertainty of the blank without substrate, and the slow rate of oxidation of substrate at low concentrations. In the case of the substrate which is oxidized most rapidly *viz.* succinate, theory is exceeded. This discrepancy appears to be due to the oxidation of products originating from the enzyme proteins by some breakdown process, and stimulated by the oxidation of added substrate. Special conditions have been found necessary to demonstrate the aerobic oxidation of oxalacetate. For one, a mixture of both pyruvate and oxalacetate must be employed, and for another, the presence of ATP appears to be required for maximal rate of oxidation. The inactivity of the catalytic system towards oxalacetate in absence of ATP may be more apparent than real. There is evidence that the following dismutation ensues:

$$2 \text{ oxalacetate} \rightarrow \text{malate} + \text{acetate} + 2\ CO_2.$$

Pyruvate formed by decarboxylation is oxidized to acetate, whilst oxalacetate is reduced to malate. Since the oxido-reduction is anaerobic, no net oxygen uptake is observable. Apparently, the aerobic oxidation of malate to oxalacetate does not proceed under the conditions of the experiment. The action of ATP in suppressing this anaerobic dismutation is not as yet understood.

Active Component of Coenzyme Concentrate. Early efforts to replace the coenzyme concentrate with known coenzymes, either singly or in combination,

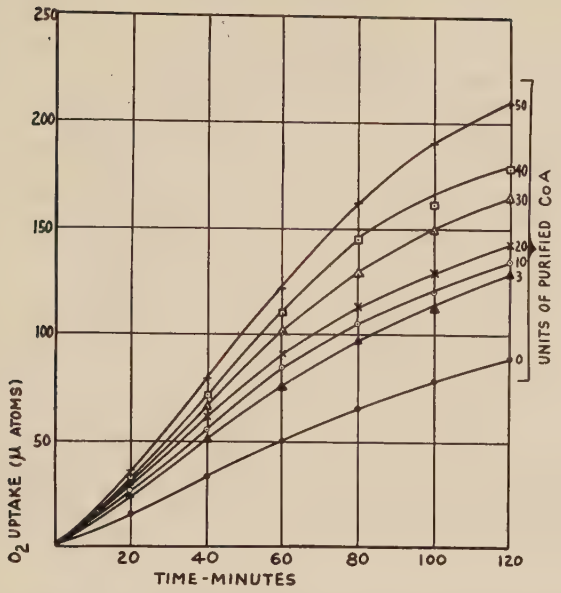

Fig. 1. Velocity of citrate oxidation as a function of coenzyme A concentration. Each manometer flask contained 0.5 ml of "5.4" enzyme, 1.0 ml. of S_{50-65}, DPN (1000 μg.), TPN (500 μg), cocarboxylase (200 μg), 2 μ moles magnesium chloride, 30 μ moles citrate, 60 μ moles glutathione (Schwarz product) and CoA as indicated. Final volume 3.2 ml.

were unsuccessful. This dilemma was resolved when we were led to investigate the effect of high concentrations of glutathione on the catalytic activity of the heart system. As shown in Table III, glutathione at relatively high levels maintains the rate of oxidation in presence of citrate for more than two hours, whereas the control rate in absence of glutathione declines after some 40 minutes. Glutathione is stable to oxidation in presence of the enzyme system, and makes little contribution to the overall oxygen uptake as shown by controls in absense of added citrate. Cysteine was inhibitory when tested at comparable concentration levels. This striking stabilization effect of glutathione opened the possibility that CoA was the active component of the coenzyme concentrate. In the crude concentrate, which is rich in CoA, there are present sulfhydryl compounds which can maintain the coenzyme at least partially in the active reduced form. In purified preparations of coenzyme A, these compounds presumably are lacking. Therefore, preparations of purified CoA do not duplicate the effect of the crude concentrates, except when supplemented with glutathione or other suitable reducing agents. Preparations of coenzyme A at a purity level of 50 per cent or higher produce a marked

Table IV

Coenzyme Requirements of Heart System.

	Oxygen uptake in 180 min. (μ atoms)
Complete System	184
no magnesium ions	134
no AMP	132
no cocarboxylase	119
no glutathione	110
no CoA	98
no DPN	93
no TPN	51

The complete system contained 0.5 ml of "5.4" enzyme, 1.0 ml of S_{50-65}, DPN (1000 μg), TPN (500 μg), 60 μ moles glutathione, cocarboxylase (100 μg), 4 μ moles AMP, 30 μmoles citrate, 2 μ moles magnesium chloride, and coenzyme A (50 units), in a final vol. of 3.5 ml.

stimulation of the activity of the heart system in presence of added glutathione. However, at a saturation level of purified coenzyme (above 17 μg of bound pantothenic acid per ml), the limiting velocity attained was still somewhat below that for crude concentrates.

Fig. 1 shows the velocity of oxidation of citrate (and subsequent oxidations) as a function of coenzyme A concentration. The velocity is proportional to the concentration of CoA over the entire range of concentrations tested. From other experiments, it would appear that the response is essentially maximal at a CoA level of about 17 units per ml (1 unit is equivalent to one μg of bound pantothenic acid).

The full component study is shown in Table IV. CoA, DPN, TPN, magnesium ions, cocarboxylase, glutathione and some as yet unidentified constituent of crude coenzyme concentrates, are all essential for maximal activity. It would appear that the crude concentrate at the levels tested, contained saturating amounts of CoA, cocarboxylase and AMP, and less than saturating amount of DPN, TPN, and sulfhydryl (glutathione?).

Oxidation of Acetoacetate. The requirements for the oxidative removal of acetoacetate are shown in Table V. At least four components are essential *viz.*: 1) the "5.4" enzyme; 2) the S_{50-65}; 3) coenzyme concentrate (DPN, TPN and CoA), and 4) some member of the citric acid cycle which can act in a sparking capacity. 2:4-Dinitrophenol at a level of 2×10^{-4} M does not inhibit the oxidative removal of acetoacetate.

TABLE V

Components for Oxidation of Acetoacetate.

	Δ Acetoacetate μ moles
Complete system	17.5
no "5.4" enzyme	1.6
no S_{50-65} fraction	0
no coenzyme concentrate	0.5
no citrate	1.0
no Mg^{++}	16.9
no cocarboxylase	14.8
with 0.0002 M 2 : 4-dinitrophenol	17.3

The complete system contained 0.5 ml of the "5.4" enzyme, 1.0 ml of S_{50-65}, 20 μ moles acetoacetate, coenzyme concentrate (5 mg), 30 μ moles citrate, 2 μ moles $MgCl_2$, cocarboxylase (100 μg), in a final vol. of 3.0 ml. Time 60 min.

TABLE VI

Conversion of $CH_3C^{14}OCH_2C^{14}OOH$ to $C^{14}O_2$.

	Acetoacetate added μ moles	Radioactivity of respiratory CO_2 c.p.m.	Recovery of original radioactivity per cent	Acetoacetate Oxidized	
				Aniline citrate method	Calculated from radioactivity of CO_2
1.	10.5	8 400	85	9.5	8.9
2.	5.4	9 350	95	5.0	5.1

The labelled acetoacetate added to each flask contained 9900 c.p.m. with a ratio of counts in the carbonyl carbon to counts in the carboxyl carbon of 0.68. Details as in legend for Table III except that 10 μ moles citrate were added as sparker. For details of the methods used in radioactivity measurements cf. (2).

The data of Table V merely show that acetoacetate disappears as determined by the aniline citrate manometric procedure (4), but do not establish directly that the disappearance is oxidative. The direct demonstration of oxidative removal is based upon experiments with carbonyl and carboxyl-labelled acetoacetate. As shown in Table VI, the recovery of some 95 per cent of the total radioactivity of acetoacetate as radioactive CO_2, makes it unlikely that any mechanism other than oxidative removal of acetoacetate was operative to any significant extent.

TABLE VII

Oxidation of $CH_3C^{14}OOH$ to $C^{14}O_2$.

	Radioactivity recovered in CO_2 per cent	Acetate oxidized μ moles
Complete system	69.5	13.5
no "5.4" enzyme	5.2	0.0
no S_{50-65}	0.1	0.0
no citrate	13.3	2.6
no coenzyme concentrate	0.5	0.1
no ATP	33.0	6.4
no Mg^{++}	30.7	5.9
with 0.0002 M 2:4-dinitrophenol	34.7	6.7

Details as for Table VI except that acetoacetate is replaced by acetate (20 μ moles) and that ATP (8 μ moles) is added in the complete system. Time 160 min. Added 222000 c.p.m. in each flask.

Oxidation of Acetate. The requirements for the oxidation of acetate are shown in Table VII. The oxidation was followed by determination of radioactivity in the respiratory CO_2 using carboxyl-labelled acetate. For maximal activity in the oxidative removal of acetate, at least five components are needed: 1) the "5.4" enzyme; 2) the S_{50-65}; 3) the coenzyme concentrate (DPN, TPN, and CoA); 4) some member of the citric acid cycle as sparker; and (5) ATP (or AMP). The last mentioned appears not to be required for the oxidative removal of acetoacetate. 2:4-Dinitrophenol at a concentration of 2×10^{-4} M inhibits the oxidation of acetate some 50 per cent.

Oxidation of Fatty Acids. The oxidation of fatty acids to carbon dioxide and water requires the following components: 1) the "5.4." enzyme; 2) S_{40-60}, and 3) DPN, TPN, CoA and glutathione (cf. Table VIII). Neither sparker nor ATP are needed for maximal activity. If anything these exert inhibitory effects. The coenzyme concentrate can be replaced quantitatively by DPN, TPN, CoA and glutathione at the levels tested.

The oxidation can be followed either manometrically by measuring oxygen uptake or isotopically by measuring the radioactivity of carbon dioxide liberated from the reaction mixture with butyrate-1-C^{14} as substrate. On the assumption that one molecule of butyrate is oxidized for each 10 atoms of oxygen absorbed, the values for butyrate oxidized, calculated from oxygen uptake, agree reasonably well with the values based on recovery of $C^{14}O_2$ (cf. Table VIII). The latter are usually lower by a constant amount which

TABLE VIII

Component Study for Butyrate Oxidation as Measured by Oxygen Uptake and $C^{14}O_2$ Formation.

	Butyrate oxidized (μ moles)	
	Based on oxygen uptake	Based on $C^{14}O_2$ formation
Complete System	6.0	5.3
no DPN	0.6	0.5
no TPN	3.6	2.6
no CoA	4.0	3.1
no glutathione	3.9	3.0
no "5.4" enzyme	0	0
no S_{40-60}	0	0

The complete system contained DPN (1 mg), TPN (100 μg), CoA (60 μg), 30 μ moles glutathione, 20 μ moles butyrate, 0.5 cc of "5.4" and 1.0 cc of $S_{40\ 60}$. Final volume 3.0 cc –38° — 120 min. incubation.

TABLE IX

Specificity of the Fatty Acid Oxidizing System.

	Relative rates of oxidation
Butyrate	100
Valerate	35
Crotonate	30
Caproate	18
Isocaproate	10
Vinyl acetate	10
Octanoate	5

Details as in legend for Table VIII.

reflects the amount of label trapped, as it were, in the form of members of the citric acid cycle. The manometric method is of course, not subject to this error.

Only the lower fatty acids and their derivatives are oxidized in this heart system (cf. Table IX). Clearly some additional enzymes or additions are needed for the oxidation of higher fatty acids.

The "5.4" suspension after repeated freezing and thawing can be separated into two fractions: 1) a soluble supernate and 2) a particulate residue.

TABLE X

Resolution of Particulate "5.4" Fraction into two Fractions.

	μ moles butyrate oxidized
S_{40-60} + 5.4 supernatant + 5.4 residue	10.4
S_{40-60} + 5.4 supernatant	1.4
S_{40-60} + 5.4 residue	0

Details as in legend for Table VIII.

TABLE XI

Oxidative Phosphorylation in the Oxidation of Succinate.

	Phosphate esterified (μ moles)	Oxygen absorbed (μ atoms)	P/O
Complete system	25.2	78.5	0.38
no ATP	0.1	91.3	0.00
no hexokinase	1.6	79.9	0.02
no succinate	0.0	0.0	0.00
no magnesium	9.8	81.5	0.13
with 0.0002 M 2:4-dinitrophenol	0.0	69.7	0.00

The complete system contained 0.3 ml of "5.4" enzyme, 8 μ moles ATP, 80 μ moles phosphate (pH 7.4), yeast hexokinase (2.5 mg), 100 μ moles fructose, 150 μ moles succinate, and 2 μ moles $MgCl_2$. Final vol. 3.0 ml. Time 70 min.

Neither of these fractions alone show any activity but both together reconstitute the original activity in presence of the other components of the system (cf. Table X). The enzymes of the soluble supernate appear to be the limiting ones for the reaction since the rate of oxidation is proportional to the concentration of the 5.4 supernate over fairly wide limits.

2:4-Dinitrophenol at a concentration of 2×10^{-4} M inhibits the oxidation of butyrate approximately 50 per cent. Fatty acid oxidation is inhibited to the same extent by this reagent as the sparked oxidation of acetate.

Oxidative Phosphorylation. The "5.4" enzyme fraction contains all the enzymatic components needed for the aerobic oxidation of succinate to fumarate. No supplements in the way either of soluble enzymes or coenzymes are needed for this oxidation. When the "5.4" fraction is linked with the glucose-hexokinase system through ATP, then the oxidation of succinate is accompanied by esterification of inorganic phosphate (cf. Table XI).

TABLE XII

Oxidative Phosphorylation During Oxidation of α-Ketoglutarate.

	Phosphate esterified (μ moles)	Oxygen absorbed (μ atoms)	P/O
Complete system	44.6	91.5	0.49
no S_{25-40}	0.0	10.0	0.00
no coenzyme concentrate, DPN or TPN	0.2	4.6	0.04
no coenzyme concentrate	3.5	31.0	0.11
no DPN or TPN	10.2	40.0	0.26
with 0.000 M 2:4-dinitrophenol	4.6	90.5	0.05

The complete system contained 0.5 ml of "5.4" enzyme, 0.5 ml of S_{25-40}, liver coenzyme concentrate (12 mg), 70 μ moles phosphate (pH 7.4), DPN (1000 μg), TPN (500 μg), 100 μ moles α-ketoglutarate, 2 μ moles $MgCl_2$ and 8 μ moles ATP.

The P/O[1] ratio uniformly approaches 0.5 as the upper limit, and as yet no way of improving the ratio has been found. Under the conditions of the experiments, fumarate (or its conversion product *l*-malate) undergoes no further change, since DPN and the S_{50-65} are required for its further removal. None of the supplements tested appeared to increase the extent of the phosphorylation accompanying the oxidation of succinate. From such results, the tentative conclusion may be drawn that the complete apparatus involved in the implementation of the oxidative phosphorylation which accompanies oxidation of succinate resides in the particles of the "5.4" fraction, though it is not excluded that some or all of the constituent enzymes are also in the soluble fraction.

The addition of soluble enzyme factors, and coenzymes to the particulate fraction, makes possible oxidative processes other than the oxidation of succinate to fumarate. It was of considerable interest to establish whether oxidative processes such as the oxidation of other members of the cycle are accompanied by esterification of inorganic phosphate. The use of the S_{50-65} fraction as a supplement is interdicted, since this fraction contains a sufficient amount of each of the enzymes of glycolysis, and a glycolytic esterification of inorganic phosphate is superimposed on that due to oxidative phosphorylation. However, the S_{25-40} fraction, which is free of most of the glycolytic enzymes, can be used as a supplement which enables the "5.4"

[1] P/O is the usual abbreviation for the ratio of moles of inorganic phosphate esterified per atom of oxygen absorbed.

enzyme fraction to carry on the rapid, two-step oxidation of α-ketoglutarate to fumarate via succinate. Some typical data are shown in Table XII. Since the P/O ratio for the oxidation of succinate to fumarate in this particular experiment was 0.32, and the over-all P/O ratio for the two-step oxidation was 0.49, it would follow that the P/O ratio for the first step (α-ketoglutarate to succinate) was between 0.6 and 0.7. While malonate can be used as an inhibitor for the oxidation of succinate, it is not an ideal inhibitor in the pig heart system in that its presence leads to rapid decline in the rate of oxidation of α-ketoglutarate.

The most characteristic property of oxidative phosphorylation, as studied in mitochondrial preparations, is sensitivity to low concentrations of 2:4-dinitrophenol (3). The sensitivity of the phosphate esterification process in the pig heart system to dinitrophenol brings this process into line with the phenomenon of oxidative phosphorylation.

DISCUSSION

Some information is now available as to which enzymes of the reconstructed system are associated with the particulate fraction and which are present in soluble form. Thus far, the following enzymes, originally associated with the mitochondrial unit, have been demonstrated to be present — at least in part — in the soluble fraction: DPN cytochrome reductase, pyruvic oxidase, α-ketoglutaric oxidase, malic dehydrogenase, isocitric dehydrogenase, aconitase, the new enzymatic component described by Gergely, Hele and Ramakrishman (5), which catalyzes the deacylation of acyl CoA and the enzymes involved in the activation of acetate, acetoacetate, fatty acid oxidation and the oxidative phosphorylation accompanying the oxidation of α-ketoglutarate. The succinic oxidase and the main components of the aerobic respiration system appear to be associated with the particulate fraction. When oxygen is replaced by other appropriate electron acceptors, all the reactions described above — with the exception of the oxidation of succinate — proceed in the soluble fraction. Thus the 5.4 fraction appears to provide merely the apparatus for interaction with molecular oxygen. This distribution of enzymes between the two fractions can be modified by appropriate techniques, such as freezing and thawing of the particulate fraction which leads to the solubilization of certain enzymes. Other communications from this laboratory have dealt with some of the particle-bound enzymes solubilized by such techniques *e. g.*, pyruvic and α-ketoglutaric oxidases, and DPN cytochrome reductase (8, 10, 11).

SUMMARY

A reconstructed non-mitochondrial system of pig heart muscle, consisting of a particulate and soluble fraction, has been shown to catalyze the complete oxidation to carbon dioxide and water of members of the citric acid cycle, acetate, acetoacetate and fatty acids. Oxidative phosphorylation has been shown to accompany the oxidation of succinate and α-ketoglutarate. The coenzyme requirements of this system have been studied.

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BRIEF NOTES

RELATION BETWEEN THE BODY WEIGHT AND THE AVERAGE DNA CONTENT OF LIVER NUCLEI IN POSTNATAL GROWTH OF THE RAT

M. FUKUDA and A. SIBATANI

Microbial Diseases Research Institute, University of Osaka, Osaka, Japan

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Recent studies on the average DNA content of cell nuclei as determined macrochemically have yielded partially contradictory data especially with adult rat livers (1, 2, 4, 5, 7, 9, 11, 12, 13, 17). This may partly be due to the variation in the frequency distribution of nuclei from different ploidies (16). The relative frequency of various nuclear types in the liver will be affected by the age even of the normal animal. Cytological and cytochemical evidence suggests that liver cell nuclei with higher DNA contents (tetra- and octoploid) are more abundant in older than in younger animals (8, 16). Thus, it is of importance to examine how the average DNA content of nuclei of rat liver varies in the course of postnatal growth, subsequent to the disappearance of hematopoietic activity. Some data were presented by Harrison (7) to this effect, but they are far from sufficient.

Determinations of the DNA content per nucleus were made essentially according to the procedure proposed by Cunningham, Griffin and Luck (2), with some modifications to be reported (6). Each figure presented in the tables represents the value obtained with a single animal. For one experiment liver was collected from five rats weighing 12 g and two rats weighing 25 g, respectively. All determinations were run in duplicate and the experimental error was found to be $\pm$ 0.25 $\mu\mu$g, but in most cases $\pm$0.1 $\mu\mu$g. The reproducibility of the data was checked from time to time and proved to be satisfactory.

The average DNA content of nuclei, and consequently the relative frequency of nuclei in different ploidy classes in the rat liver, is highly dependent on the body weight of animals growing under ordinary nutritional conditions, as revealed by determinations in various stages of postnatal growth. Table I shows that the average DNA content of liver nuclei considerably increases between 25–100 g body weight, but thereafter hardly changes at all. The increase may largely be due to the polyploidization of parenchymatous cell nuclei. This postulate is fully supported by microspectrophotometry of Feulgen-stained materials with the improved optical arrangement of Naora (10). According to this study (14), there are few nuclei showing DNA content higher than the postulated diploid value of Class I (*cf.* 16) nuclei in livers of 12 and 25 g rats; it is at the body weight around 50 g

that an extensive transition from Class I (diploid) nuclei to Class II (tetraploid) nuclei occurs in the liver; in rats of the body weight higher than 100 g the highest peak in the histogram of nuclear DNA content always appears in Class II.

Meanwhile, a slight decrease in the average DNA content of nuclei between 12 and 25 g rats merits special attention. Our method of determination is sufficiently sensitive to disclose the small but significant difference between these stages. The possibility that this difference is due to the presence of immature blood cells in 12 g rat livers could be ruled out from microscopic observations. Nor can it be caused by a specifically increased mitotic activity in either of the stages concerned. The noted difference may rather be attributed to the difference between the DNA contents of the diploid hepatic nuclei *per se* in the two stages under consideration. It should be kept in mind that microspectrophotometric determinations (14) have

TABLE I

Average DNA content of rat liver nuclei ($\mu\mu$g) during postnatal growth.

Body weight in g mean and range	Sex	Animals[1]			Mean
		I	II	III	
12 (11.9–12.3).....	—	5.82	5.81	6.08	5.90
25 (24.9–25.2)	—	5.03	5.18	5.07	5.09
49 (48– 51).....	♂	9.42	9.53	9.28	9.41
51 (49– 52).....	♀	9.18	9.09	9.14	9.14
97 (96–100).....	♂	11.1	11.2	11.0	11.1
98 (96–100).....	♀	11.2	10.8	11.3	11.1
198 (196-200).....	♂	11.4	10.8	11.5	11.2
200 (200).....	♀	11.0	11.6	11.2	11.3
338 (320–355).....	♂	11.3	11.3	11.5	11.4

TABLE II

Average DNA content of liver nuclei of adult rats subjected to prolonged starvation and of young rats the growth of which was retarded by restricting caloric intake.

Prolonged starvation					Growth retardation			
Sex	Initial body weight g	Per cent loss of body weight	Period of experiment day	DNA per nucleus $\mu\mu$g	Sex	Final body weight g	Period of experiment day	DNA per nucleus $\mu\mu$g
♂	190	32	7	11.5	♂	49	26	9.26
♂	200	33	9	11.7	♀	51	28	9.26
♂	210	38	11	10.5	♀	51	30	9.50
			mean	11.2			mean	9.34

[1] Values of each body weight group are arranged from I to III according to the increase of the body weight of individual animals used.

shown an unmistakable decrease in the nuclear DNA content of the liver parenchyma between the body weights of 12 and 25 g.

Experiments with fasting and malnutrition supply further evidence that the average DNA content of liver nuclei in animals of a given body weight is constant (Table II). Rats weighing originally about 200 g were rendered moribund by prolonged starvation and then subjected to analyses. No significant decrease from the normal value of 200 g rats was observed in the DNA amount per nucleus. It can thus be stated that the average DNA content of liver nuclei is hardly affected even by prolonged starvation. Essentially similar results are already announced by some workers (3, 9), but the actual details of the experiments seem so far to be unpublished.

Experiments with malnutrition were made on young rats weighing about 40 g, the growth of which was inhibited by restricted caloric intake for four weeks. During this period the body weight of the animals only gained 10 g, whereas, under normal nutritional conditions, it would have gained 100 g. Comparison of DNA per nucleus of the liver among these animals, now weighing 50 g, and normally growing ones of the same body weight makes it clear that this value is not dependent on the age, nor on nutritional conditions, but on the body weight of the animals. It can now be assumed that also in liver of actively growing rats, the frequency distribution of nuclei in different degrees of ploidy depends directly upon the body weight. It appears that the higher values of DNA per nucleus obtained by Ely and Ross (5) with liver from rats fed on low protein diets, do not depend on their employing younger animals, as suggested by Campbell and Kosterlitz (1).

Summary. During the earlier course of the postnatal growth of the rat, the average DNA content of liver nuclei as determined macrochemically is a function of the body weight. Experiments on prolonged fasting and malnutrition demonstrate that this value is determined by the maximum body weight which the animals have attained irrespective of growth rate or nutritional conditions.

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AUTORADIOGRAPHS OF ALGAE AND CILIATES EXPOSED TO TRITIATED WATER[1]

CICILY CHAPMAN-ANDRESEN

Cytochemical Department, Carlsberg Laboratory, Copenhagen, Denmark

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Eidinoff, Fitzgerald, Simmel and Knoll (4) and Fitzgerald, Eidinoff, Knoll and Simmel (5) have used tritium for autoradiographic studies on *Paramecium* and yeast, grown in aqueous media containing sodium acetate, labelled with tritium, and have pointed out the advantages of this isotope for such investigations.

In the experiments reported here, an attempt was made to obtain food organisms, labelled with tritium, for ingestion by the amoeba *Chaos chaos* for studies comparable to those described by Andresen, Chapman-Andresen and Holter (1) with ^{14}C. Chlorophyll-containing and chlorophyll-free organisms were used, the latter feeding on bacteria; experiments are now in progress with ciliates (*Tetrahymena geleii*) grown on a synthetic medium.

Experimental. Organisms: The chlorophyll-containing organisms were the alga *Scenedesmus* sp. and the ciliate *Paramecium bursaria*, which contains symbiotic algae. The chlorophyll-free organism used was a ciliate, *Colpidium* sp. from mixed cultures containing bacteria.

Solutions: Tritiated water, containing 200 mC per ml (from the Atomic Energy Research Establishment, Harwell) was diluted with an aqueous solution of salts to give the salt concentration described by Österlind (6). The solutions used for the culture of the organisms contained 3–33 mC per ml (ca. 0.6 6×10^7 disintegrations per minute per μl). Eidinoff *et al.* (4) used solutions the activity of which was of the same order of magnitude as those used here, for yeast "the activity of the water and sodium acetate in this medium were 2×10^6 and 4×10^7 disintegrations per minute per mg respectively." During exposure to tritium, the organisms were kept in sealed glass ampoules for 48 hours to 4 weeks; organisms containing chlorophyll were kept under constant light (see (1)).

Autoradiographic technique: After exposure to tritium, the organisms were washed in Österlind solution and smears of *Scenedesmus* and *Colpidium* were made on gelatine-alum treated slides (3). Specimens of *Paramecium bursaria* were placed singly in small drops of water on a slide and torn open with a fine glass needle to separate the algae and nuclei. The organisms were not fixed, but allowed to dry in air.

Kodak Autoradiographic Stripping Emulsion was used with the technique de-

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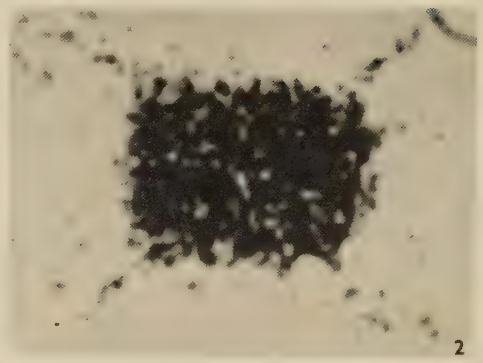

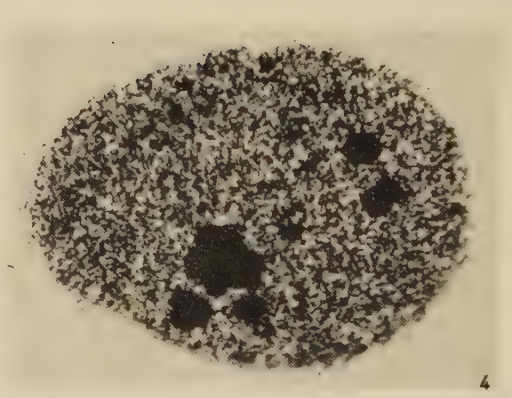

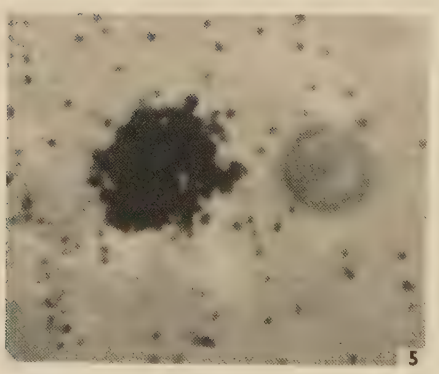

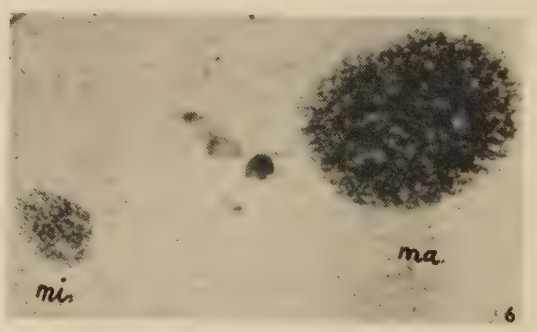

Fig. 1. Photograph of living *Scenedesmus*; the processes at the corners of the parcel of 4 cells can be clearly seen. 1800 ×.

Fig. 2. Autoradiograph of *Scenedesmus* from smear, after 48 hours in tritiated water, containing 33 μC per μl. Exposure 7 days. The processes at the corners of the cells are clearly visible. 1800 ×.

Fig. 3. Autoradiograph of *Scenedesmus* from smear after 48 hours in a solution containing ^{14}C as carbonate. Activity of solution 2×10^{-3} μC per μl. Exposure 24 hours. 1800 ×.

scribed by Doniach and Pelc (3), stored during exposure in light-tight boxes at 4° C. and processed as described in (1), but without restripping. Smears and squashes of organisms subjected to the same conditions, but without tritium, showed no artefacts due to chemography or pressure.

Discussion. The experiments show that tritiated water is taken up by photosynthesizing organisms (figs. 2 and 5) and that it is also present in the nuclei of *Paramecium bursaria* (fig. 6). Eidinoff *et al.* (4) found indications of the macronucleus in sections of *Paramecium aurelia*, and in the squashes illustrated here, autoradiographs of both macronucleus and micronucleus are recognizable by their shape and size; the actual nuclei could not be identified in the squash, since development had damaged the cytological details (1). Food vacuoles are visible in autoradiographs of *Colpidium*, and in addition, considerable activity is distributed over the whole of the cytoplasm (fig. 4). The power of resolution, stated by Eidinoff *et al.* to be at least 1 μ, is illustrated here by the presence in the autoradiograph of *Scenedesmus* (fig. 2) of the 1 μ wide processes at the corners of the cell parcel (compare with the resolution in the autoradiograph of *Scenedesmus* containing ^{14}C (fig. 3)).

These results indicate that the tritium found in the organisms participates in photosynthesis (*Scenedesmus* and the symbiotic algae of *Paramecium bursaria*) or is introduced by way of the food (*Colpidium* and *Paramecium bursaria*) and not only by direct diffusion of tritiated water.

All organisms died within 24 hours of exposure to a solution of tritiated water, containing 167 mC per ml. Powers and Schefner (7) reported the survival of *Paramecium aurelia* after exposure for two days to solutions with 1–100 mC of tritium per ml; division occurred during this period, but the death rate during autogamy was increased. *Colpidium* cultures were grown in a medium containing tritiated water in a concentration of 33 mC per ml for four days without any obvious deleterious effects, but when *Paramecium bursaria* was kept for two days in the same concentration, all organisms died within 18 days after removal from the active solution; four days after exposure to tritium the number of symbiotic algae had fallen to about 10 per cent of the normal value. This effect has been observed during rapid division and after X-ray treatment by Wichterman (8) and after exposure to solutions containing ^{14}C (2). *Scenedesmus* survived the same treatment for four weeks.

The experiments reported here thus demonstrate an uptake of tritium from tritiated water by unicellular organisms and a variation in sensitivity of the different organisms to the radiation, a fact well known for various other types of radiation.

Fig. 4. Autoradiograph of *Colpidium* from smear, after 48 hours in tritiated water, containing 33 μC per μl. Exposure 7 days. Food vacuoles can be seen, otherwise the activity seems evenly distributed throughout the cytoplasm. 600 ×.

Fig. 5. Autoradiograph of a single symbiotic alga from a squash of *Paramecium bursaria*, after 48 hours in tritiated water, containing 33 μC per μl. Exposure 7 days. The autoradiograph became displaced during processing, and lies slightly above and to the right of the alga, it is somewhat larger than the alga owing to stretching of the emulsion. 2200 ×.

Fig. 6. Autoradiograph of part of a squash of *Paramecium bursaria*, after 48 hours in tritiated water, containing 33 μC per μl. Exposure 7 days. The macronucleus (to the right) and the micronucleus (to the left) can be clearly seen. 540 ×.

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ISOLATION AND SOME ENZYMIC PROPERTIES OF EHRLICH ASCITES TUMOR MITOCHONDRIA

O. LINDBERG, M. LJUNGGREN, L. ERNSTER, and L. RÉVÉSZ

Wenner-Grens Institute, University of Stockholm and the Wallenberg Laboratory, Karolinska Institutet, Stockholm, Sweden

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The behavior of aerobic energy-generating processes in neoplastic tissue has long been the subject of intense studies, on levels varying from the intact cell to the single enzymes (19, 6). The development of the technique of cell fractionation by differential centrifugation (16) and the recognition of mitochondria as a site of the above mentioned processes (5) afforded a new promising basis for continued work in this field. Morphological (3, 1), chemical (16, 7) and enzymic (13, 14, 18, 20) data as yet available unequivocally indicate that there *are* deviations from the normal pattern in tumor mitochondria (the term "normal pattern," however, not including in most instances the embryonic one), but this does not mean that these deviations fit into a unitary syndrome.

Systematic investigations of this kind may greatly be facilitated by availability of pure neoplastic cultures. The Ehrlich ascites tumor of mice, intensely studied and characterized during the last years by Klein (10) seems to present an ideal material. It consists of a freely suspended cell population, easily available with not more than 10 per cent of non-neoplastic contamination. In this report we describe a method for isolation of mitochondria from this material, with some data about the capacity of these mitochondria to oxidize members of the Krebs cycle and to exhibit coupled phosphorylation.

Experimental. The preparation of mitochondria was based upon the classical principles of differential centrifugation by Schneider and Hogeboom (16). Due to the special nature of this material, homogenization was performed with quartz sand. As a preparing medium (referred to below as sol. S.) 0.25 M sucrose was employed, containing 0.01 M sodium β-glycerophosphate buffer (pH 8.2) and 0.01 M versene of which the latter is a powerful protecting agent for mitochondria (17).

25 ml of ascites punctate from two tumor bearing mice (11 days old inoculates) were centrifuged. All operations thereafter were performed in the cold. The cell mass (c. 8 ml) was washed four times with three volumes of sol. S. The washed cells, suspended in an equal volume of sol. S., were homogenized by vigorous shaking with c. 1/3 vol. of quartz sand (Merck) in a glass-tube. Centrifugations were performed in an International Refrigerated Centrifuge with a multispeed attachment at 0° C. The homogenate was diluted with 10 ml of sol. S. and centrifuged at 1000 r.p.m. for five minutes. The elution was repeated five to seven times. The combined supernatants were centrifuged at 15 000 r.p.m. for 15 minutes. The mitochondrial residue was washed once by gentle suspension in 10 ml of sol. S.,

TABLE I

Oxidative Phosphorylation in Ehrlich Ascites Tumor Mitochondria with Various Substrates.

Substrate	O_2 consumed	P esterified	P/O
	microatoms per mg protein-N	micromoles per mg protein-N	
none	0.0	0.0	—
pyruvate	1.4	1.7	1.2
pyruvate + 1 μM succinate	4.8	9.7	2.0
citrate	0.3	1.3	—
α-ketoglutarate	5.3	9.6	1.8
succinate	8.6	11.8	1.4
malate	3.9	7.8	2.0

Each Warburg vessel contained: mitochondria, 1.51 mg protein-N; substrate, 20 μM; orthophosphate, 40 μM; adenylic acid, 2.7 μM; Mg^{++}, 9 μM; KCl, 90 μM; glycylglycine buffer (pH 7.8), 45 μM; glucose, 140 μM; hexokinase, V_{max} = 35 μM/min. Final volume 1.5 ml. Centre well: KOH. Side arm: H_2SO_4 (see text). Gas phase: air. Temp. 30° C. Duration of experiment 37 minutes.

and recentrifuged. The preparation was examined under the phase contrast microscope and by staining with Janus Green B. The preparation contained 11.3 mg protein-N as determined with the biuret reaction (15).

In order to test oxidation and phosphorylation, the mitochondria were incubated aerobically in Warburg vessels in the presence of various substrates, orthophosphate (labelled with P^{32}) and adenylic acid. The ATP formed was trapped by the glucose-hexokinase system. Hexokinase was prepared according to Berger *et al.* (2) (step 5) with minor modifications (11). The substrates used were: pyruvate, pyruvate +1 micromole succinate, citrate, α-ketoglutarate, succinate and malate. After seven minutes of thermo-equilibration the manometer taps were closed and respiration measured under 30 minutes. The samples were fixed by tipping in 0.2 ml 10 N H_2SO_4 from the side arm. An aliquot of 0.2 ml was removed from each vessel and transferred into a two-phase-mixture of isobutanol-benzene and molybdic acid for phosphate determination according to Martin and Doty (12). In this method orthophosphate is recovered in the organic layer of the mixture, while the organically bound phosphate is left in the water phase. By determining the ratio of the number of counts found in the both phases (4) the amount of esterified phosphate can be calculated with great accuracy, even if the esterified phosphate is a very low percentage of the total.

In Table I the oxygen consumption and phosphate uptake for the various substrates are given in terms of mitochondrial nitrogen. The oxygen values are corrected by extrapolation for the equilibration time. The yield of coupled phosphorylations is expressed in terms of micromoles of phosphorus per microatoms of oxygen (P/O ratio). It is seen from the table that both respiration and phosphorylation

are very low. The rate of phosphate uptake in terms of micromoles P per mg N per minute ranges between 0.05–0.3. The value is about 2 for normal mouse liver (9) and about 4 for rabbit heart (11). The P/O ratios are also somewhat depressed as compared with the generally accepted values for normal tissues (8). Succinate possibly forms an exception giving a rather normal P/O ratio. It may be noticed, furthermore, that pyruvate without "sparker" gives a low but significant respiration; addition of a catalytic amount of succinate is strongly stimulatory. Citrate, on the other hand, is oxidized at a very low, if at any measurable, rate. More detailed investigations are in progress.

We wish to express our gratitude to Dr. G. Klein for supplying us with the tumor material.

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HEAVY METALS, FERTILIZATION AND CLEAVAGE IN THE EGGS OF *PSAMMECHINUS MILIARIS*

K. W. CLELAND

Zoology Department, University of Cambridge, England and Marine Station, Millport, Scotland

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During work on the eggs of *Psammechinus miliaris*, some difficulty was experienced in making successful fertilizations. The following grades of abnormal fertilization were encountered: 1) the eggs responded by a transient surface contraction which occurred 1–2 minutes after insemination, but no fertilization membrane was elevated; cleavage was grossly abnormal in that the furrows either did not form or appeared as fine irregular slits which not infrequently disappeared later; 2) after a more marked surface contraction, the fertilization membrane separated over a small area of the egg surface and caused a marked indentation of the underlying egg; with subsequent rounding up of the egg the space between membrane and egg was obliterated; cleavage abnormalities were usually as in 1); 3) membranes separated after a short delay but were not concentric with, and caused some indentation of the egg during elevation; cleavage was either normal or somewhat polyspermic.

Multiple sperm asters could also be seen in eggs showing abnormalities of grades 1) and 2). Judging from a study of nicotine-induced polyspermy, the degree of polyspermy in the above eggs was not sufficient to account for the cleavage abnormalities, although it undoubtedly contributed to them. A great part of the cleavage abnormality is probably due to mechanical interference with cleavage furrow intrusion, caused by the closely adherent vitelline membrane.

Although a similar or identical condition to that described above has been ascribed to an underripeness of the eggs (7, 8), the mode of occurrence in the present case suggested that some toxic substance in the water was responsible. To test the possibility of a heavy metal toxicity, the effect of adding the powerful heavy metal-chelating substance versene (ethylene diamine tetraacetate (11) to the water was studied. With the versene an amount of sodium hydroxide was added equivalent to the hydrogen ions released by the chelation of the magnesium in the sea water.

At concentrations of versene in excess of 2.5×10^{-5} M, fertilization and cleavage were normal and normal plutei developed; in the absence of versene, abnormalities of grade 1) were found. Provided the versene was added before the surface contraction was maximal, normal membrane elevation and cleavage followed insemination. Added after this time versene was without effect on the abnormalities, which were also unaffected by reinsemination. No toxic effects of versene were found in concentrations of up to 10^{-3} M, the highest used.

These abnormalities of fertilization and cleavage were all encountered while using sea water drawn from the laboratory supply. This had previously been satisfactory. The fact that normal fertilization and cleavage resulted when the eggs were fertilized in water drawn directly from the sea, indicated that versene was not having a direct effect on the egg unrelated to the removal of heavy metals.

The laboratory sea water supply comes into contact with two principal metals—copper (in the taps) and lead (in the main pipe-line). The possibility that the lead pipes are alloyed with some other metal (zinc or tin) must, however, also be considered. Analyses of the toxic water for copper, lead (2) and zinc (10) were carried out and indicated the presence of concentrations of less than 10^{-7} M, 2.5×10^{-6} M and 2×10^{-5} M respectively. The effect on fertilization and cleavage of various concentrations of cupric sulphate, lead nitrate and zinc sulphate, added before fertilization to suspensions of jelly free eggs in normal sea water, was then studied.

Copper sulphate additions had the following effects: 1×10^{-5} M—no sperm penetration; 5×10^{-6} M—no membrane elevation but mostly normal and polyspermic cleavage; 2.5×10^{-6} M—excentric membrane elevation and normal cleavage; 1×10^{-6} M—no effect. A concentration of added lead of 6×10^{-4} M was required to suppress membrane elevation; at this concentration of lead the subsequent cleavages were mostly normal and polyspermic although some were like those found in the toxic sea water. Zinc additions caused the following effects: 1×10^{-4} M—no sperm penetration; 1×10^{-5} M—abnormalities typical of grade I) above; 2.5×10^{-6} M—abnormalities of grades 2) and 3). When zinc sulphate (1×10^{-5} M) was added to suspensions of eggs in which the fertilization membranes were already elevated (30–60 seconds after insemination), no abnormality other than slight cleavage delay was observed.

The experiments described above indicate that the toxic effects of the laboratory sea water are due to its contamination with zinc. Zinc is the only metal of those tested which gives results qualitatively identical with those found in the toxic water and is the only metal in the toxic water present in biologically effective concentration. As would be expected from the analytically determined zinc content, when the toxic water was diluted 4-fold with normal sea water, its effect on fertilization and cleavage was identical with that found in sea water to which a concentration of 5×10^{-6} M zinc sulphate had been added. The concentration of versene needed to detoxicate the toxic water is also consistent with the zinc content of the water as determined by analysis.

Analyses for zinc of sea water taken from various points of the laboratory installation indicated that a toxic concentration of zinc was reached only when the water had been in contact with the pipes for a day or so. The sudden appearance of the toxicity during the present work was apparently due to a temporarily reduced flow of water through the particular section of the piping concerned.

An inhibition of membrane elevation in sea urchin eggs by copper salts has been described by previous workers (3, 4, 6); Hoadley (3) also found lead and zinc to be inhibitory. The concentration of copper found to prevent sperm entry in the present work also causes an increased respiratory rate in the sperm of *Echinus esculentus* (5). Although the abnormalities found in underripe eggs (7, 8) are evidently not due to heavy metals (1), it is interesting that substances which counter-

act the underripeness—proteins and amino acids (9, 12)—are also able to react with heavy metals. Underripeness as such does not appear to occur in the eggs from Millport sea urchins.

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NUCLEIC ACIDS ASSOCIATED WITH THE NUCLEOLI OF LIVING SEGMENTED RAT EGGS

C. R. AUSTIN

Division of Animal Health and Production, C.S.I.R.O., McMaster Laboratory, Sydney, Australia

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In the course of a study on the early segmentation of the rat egg, an irregularly shaped but clearly defined aggregation of material could often be seen, with the phase-contrast microscope, about the nucleoli of living eggs. This material, referred to for convenience as the "peri-nucleolar material," could not be discerned in the pronuclei or in the nuclei of 2-cell eggs; in 4-cell eggs it could generally be found, though only in the form of small localized projections (Fig. 1). In 8- to 16-cell eggs the peri-nucleolar material was much more evident and often appeared as lobes on either side of the nucleolus (Figs. 2 and 4) or as a complete though irregular ring about the nucleolus (Fig. 3). The apparent density of the material varied from a thin film to a mass so dark that the limits of the nucleolus could scarcely be discerned.

Observations were extended by the use of an ultra-violet microscope, consisting of reflecting condenser and objective, each of 3 mm. focal length and N.A. 0.78.[1] Wave length was selected by means of a Beckman monochrometer, and the standard Beckman hydrogen lamp was used as the source of radiation. With an initial magnification for photography of $\times 330$, a satisfactory exposure with Kodak "Ortho X" plates was 6 minutes. Photographs were taken with a slit width of 2 mm., which, in the region of 260 mμ, gives a wave length range of ± 2.5 mμ. Preliminary tests showed that living eggs could tolerate about 20 minutes irradiation at this wave length without showing any observable change in structure or absorption characteristics.

By this means it was found that the absorption in the region of 260 mμ was fairly uniformly distributed in the nucleus and cytoplasm of 2-cell eggs, but in 4-cell eggs there was greater absorption by the nucleus and particularly by a ring of material about the nucleolus. This effect was more clearly shown by 8-cell eggs, in which the absorption immediately around the nucleoli was very high.

Eggs in the 8-cell stage were then photographed, first with the aid of the phase-contrast microscope and then in the ultra-violet with the apparatus described above. The two photographs were taken as nearly in the same focal plane as possible. The results showed a close correspondence between the peri-nucleolar material, as seen by phase-contrast, and the regions of greatest absorption in the ultra-violet (Figs. 5, 6 and 7). Clearly, the peri-nucleolar material represents a zone in which there is a high concentration of nucleic acids, and this concentration in-

[1] These optical parts were constructed by the Division of Physics, C.S.I.R.O.

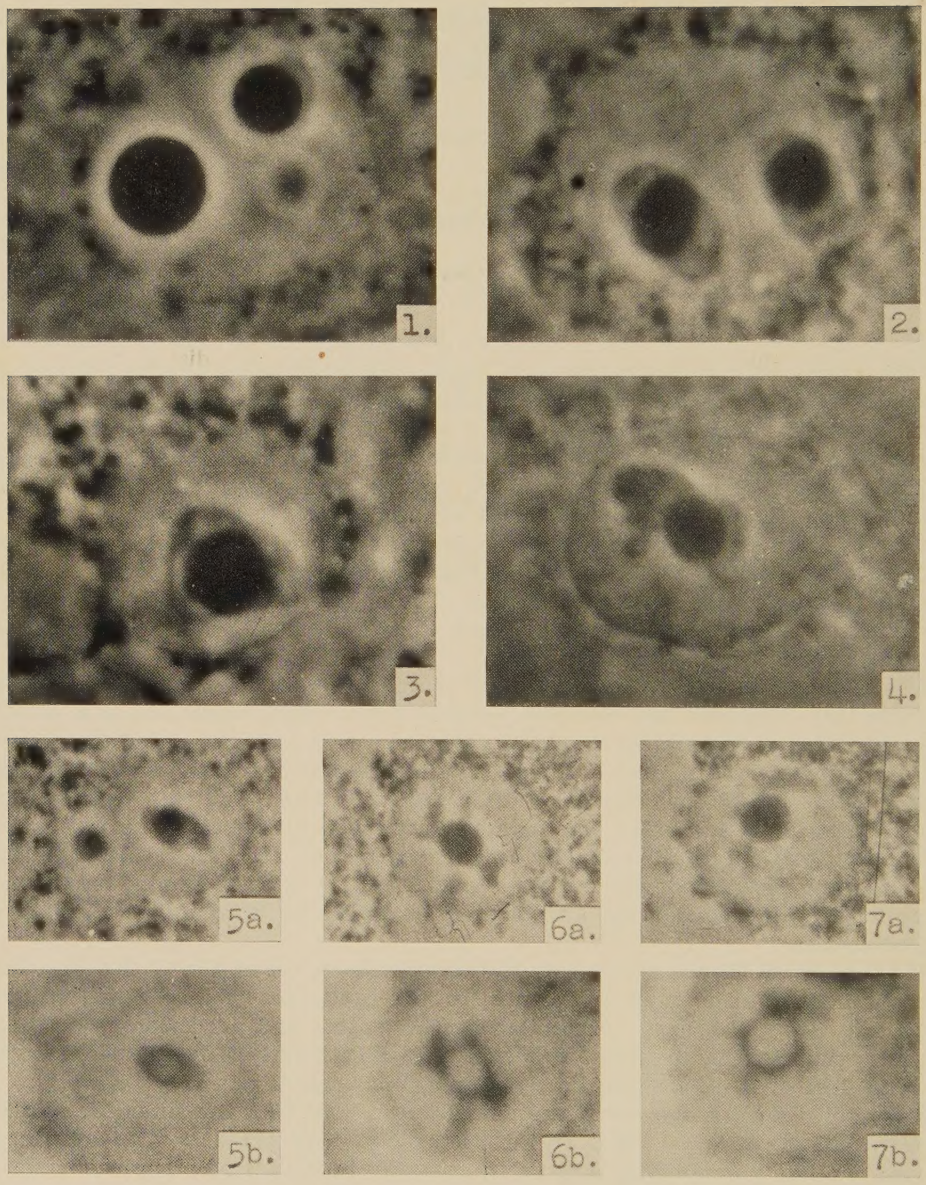

Fig. 1. Nucleus of 4-cell egg. Phase-contrast. ×2450.
Figs. 2 and 3. Nuclei of 8-cell eggs. Phase-contrast. ×2450.
Fig. 4. Nucleus of 16-cell egg. Phase-contrast. ×2450.
Figs. 5, 6 and 7. Nuclei of 8-cell eggs. (a) Phase-contrast. (b) Ultra-violet, 260 mμ. ×1150.

creases as nucleolar surface area decreases with successive segmentations of the egg. An increasing nuclear concentration of desoxyribonucleic acid was observed by Lison and Pasteels (3) in the early segmentation of *Paracentrotus* eggs and Alfert (1) reported evidence of a similar process in mouse eggs. Furthermore, a strongly positive Feulgen reaction has been observed immediately about the nucleoli in fixed 4-cell and 8-cell eggs of the rat (Braden and Austin, unpublished observations). It seems likely, therefore, that the perinucleolar material has a high content of desoxyribonucleic acid and that it corresponds to the "nucleolus-associated chromatin" of Caspersson (2). It is of particular interest that when the concentration of nucleic acids becomes sufficiently high, as in 8-cell and later eggs, the form and distribution of the perinucleolar material may be studied in living eggs by means of phase-contrast microscopy.

A more complete report of investigations on nucleic acid distribution in rat eggs is being prepared for publication elsewhere.

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